DIINDOLYLMETHANE ANALOGS AS NOVEL NR4A1 ANTAGONISTS AND AS A NOVEL CLASS OF ANTICANCER AGENTS AND SP TRANSCRIPTION FACTORS AS NONONCOGENE ADDICTION GENES THAT ARE TARGETS OF ROS INDUCING AGENTS

A Dissertation

by

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ABSTRACT

The orphan nuclear receptor 4A1 (NR4A1) and specificity protein (Sp) transcription factors (TFs) are both overexpressed in the majority of solid tumors. Our laboratory has researched the molecular mechanisms of a novel class of 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methanes (C-DIMs) as NR4A1 antagonists and Sp proteins as non-oncogene addiction genes (NOA) that are targets of reactive oxygen species (ROS) inducing agents. Nr4A1 antagonists (DIM-C-pPhOH) (C-DIM 8) and (DIM-C-pPhCO_2Me) (C-DIM 14) inhibited cancer cell proliferation, induced apoptosis, and inhibited migration. The NR4A1 antagonists inhibited constitutive and TGFβ-induced migration in Triple Negative Breast Cancer (TNBC) cells. We also demonstrate that p38α is necessary and sufficient for TGFβ-mediated migration and NR4A1 nuclear export in triple negative breast cancer (TNBC) which was attenuated with NR4A1 antagonists (C-DIMs), leptomycin B, and the p38 inhibitor SB202190. We also demonstrate that NR4A1 is essential for TGFβ-induced EMT and NR4A1 antagonists promoted cytosolic sequestration of the transcription factor β-catenin and its proteasome-dependent degradation in a time dependent manner. β-catenin, along with TCF-3, TCF-4, and LEF-1 binds to TCF/LEF response elements in the NR4A1 promoter, regulating its expression.

RNA interference (RNAi) demonstrates that Sp1, 3, and 4 TFs individually play a role in cancer cell growth, survival and migration/invasion in cancer cell lines. Individual knockdown of Sp1, Sp3, or Sp4, resulted in inhibition of cell growth, migration, and induction of apoptosis, with no compensation. Moreover, tumor growth in athymic nude mice bearing pancreatic cancer xenografts was significantly attenuated in cells depleted of Sp1, Sp3, and Sp4 in combination or Sp1 alone. Ingenuity Pathway Analysis (IPA) of changes in gene expression in Panc1 pancreatic cancer cells after individual knockdown of Sp TFs demonstrates that Sp1-, Sp3- and Sp4-regulated genes were associated with pro-oncogenic activity.

C-DIMs are promising anticancer agents in NR4A1-overexpressing solid tumors and represent a novel class of mechanistic-based drugs that target TGFβ/NR4A1-dependent inducible migration in TNBC. The functional and genomic results coupled with overexpression of Sp transcription factors in tumor vs. non-tumor tissues and decreased Sp1 expression with age indicate that Sp1, Sp3 and Sp4 are non-oncogene addiction (NOA) genes and are attractive drug targets for individual and combined cancer chemotherapies.

DEDICATION

I would like to dedicate this thesis to my mother, who passed away May 30, 2016. She was an inspiration, a truly loving and selfless human being, an angel, and my hero. I would not be here or the man I am today without her or my dad. I would also like to thank my brother and sister, and my cousin, who is my best friend and brother for life, Ed. I would also like to thank my fiancée Ashley Hope Seifert, who has been with me through thick and thin, and times I had truly struggled with pain and loss this year. She is also my inspiration, my hero, my angel and I love her dearly with all my heart.

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CHAPTER I

INTRODUCTION

Cancer

Cancer is a complex disease that is a result of normal cells becoming transformed into a cancerous cell that displays uncontrolled cell growth, proliferation, survival, altered metabolism, and evading apoptosis (1). Cancer cells begin as a benign neoplasm that may proliferate uncontrollably but still retain normal cell physiology and phenotype (1). These cells subsequently obtain mutations, resulting in a more malignant and aggressive phenotype; this is accompanied by changes in their morphology, invasion of the surrounding tissues, intravasation of blood vessels, and then colonization of distal organs through a process that is called metastasis (2). Cancer cell metastasis results in the majority of cancer cell deaths. Cancers are broadly classified into two major types: solid tumors and non-solid malignancies. Solid tumors consist of carcinomas (cancers which arise from cells derived from cells that are endodermal in origin) sarcomas (cancers that arise from mesodermal cells and are mesenchymal in origin) and neuroendocrine tumors which arise from cells of the neuroendocrine system. Non-solid tumors encompass cancers that arise from hematological or lymphatic system malignancies, which include leukemias and lymphomas respectively (3,4). These can arise from fluid filled cystic lesions commonly associated with glands,

with proliferation of immature poorly differentiated leukocytes, granulocytes and lymphocytes and these cancer cells are dispersed and circulate throughout the body (4,5). These cancers are usually treated with ionizing radiation, chemotherapies that contain arabinoside-based nucleotide analogs and specialized chemotherapies that target cancer specific epitopes (6). For instance, non-Hodgkin's lymphoma (NHL) is normally treated with the CHOP (cyclophosphamide, hydroxydaunorubicin, oncovirin, and prednisone) chemotherapeutic regimen along with the monoclonal antibody rituximab, which specifically targets CD20 a common marker expressed on the surface of NHL cells (6,7). Other leukemias, such as chronic myelogenous leukemia (CML), are generated by chromosomal translocations (8). CML is caused by the Philadelphia chromosome which is a result of a reciprocal translocation between chromosome 9 and 22, designated t(9;22)(q34;q11) (8). This gives rise to the BCR-ABL1 fusion gene and promotes the ABL1 kinase proto-oncogene to an oncogene and driving CML progression (8,9). This cancer is treated with Gleevec (imatinib) which is a tyrosine kinase inhibitor specifically developed to treat this cancer. Solid tumors can be surgically removed if localized to specific tissue, treated with ionizing radiation, or treated with available chemotherapeutic regimens (9). Estrogen receptor positive (ER+) breast cancer can be treated with an ER antagonists such as tamoxifen, aromatase (the enzyme which converts androgens into estrogens, such as estradiol (E2)) inhibitors like anastrazole, along with other drugs including the thymidylate synthase inhibitor 5-fluoruracil (5-FU) and Adriamycin

(doxorubicin) (10). Other cancers such as glioblastoma multiforme (GBM), are very difficult to treat not only because of their location, but also the blood brain barrier (BBB) makes drug delivery very challenging (11). Drugs such as temozolomide (TMZ, a DNA alkylating agent), EGFR inhibitors (gefitinib, erlotinib, specifically targeting $EGFR_{vii}$) (classical GBM), PDGF inhibitors (i.e. crenolanib) (proneuronal GBM), carmustine (bis-chloroethylnitrosourea or BCNU, another alkylating agent), and bevacizumab (a VEGF inhibitor) (11,12). GBM is also a disease with a strong genetic component. Along with EGFR, these tumors exhibit aberrant expression of other genes, which designate their type. Classical GBM is associated with EGFR, proneuronal (platelet derived growth factor receptor α (PDGFA) isocitrate dehydrogenase-1 (IDH1)), the mesenchymal subtype (neurofibromin 1 (NF1)), and the nueral type (gamma-aminobutyric acid type A receptor alpha1 (GABARA1), Neurofilament light polypeptide (NEFL), Synaptotagmin-1 (SYT1), and solute carrier family 12 member 5 (SLC12A5). DNA methylation is also of interest in GBM and hypomethylation is associated with enhanced malignancy (13). The enzyme o-6-methylguanine-DNA methyltransferase (MGMT) is involved in repair of DNA alkylation and is a so called suicide DNA repair enzyme since the reaction is irreversible and the active site of the enzyme cannot be regenerated (14). In GBM hypermethylation of this promoter leads to decreased activity (i.e. TMZ) which enhances the response to TMZ treatment (15). Other genetically linked diseases such as Li Fraumeni syndrome (TP53) (16), Tuberous sclerosis (TSC1/2) (17), Von Hippel-Lindau

syndrome (pVHL-1) (18), Neurofibromatosis (NF-1) (18), and Turcott syndrome (MutL homologue 1 and 2 (MLH1 and MLH2) and MutS homolog 6 (MSH6), and endonuclease PMS2 (19), all of which are critical in the DNA mismatch repair (MMR) system).

Cancer mortality figures

Cancers are among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2016 (20). Cancer related deaths have continued to rise over the past decades and this emphasizes the necessity for development of more innovative, efficacious, less cytotoxic, and targeted chemotherapeutic agents, especially for late stage/ metastatic tumors (20). In 1990, 5.8 million people died of cancer in the worldwide and in 2010, this number has increased to 7.8 million (21). Lung, colorectal, breast, and prostate cancers are the most commonly diagnosed cancers (21). In 2012, lung cancer has the highest mortality rate associated with all cancer related deaths (1.59 million, 20.2% of all cancer deaths), followed by liver (745,000 9.7%), stomach (723,000, 9.2%), breast (521,000, 6.4%) and esophageal (400,000 5.0%) cancers (21). Liver, esophageal and pancreatic cancers have the highest mortality rates, and for pancreatic there is a 95% likelihood of mortality within a year (21). This is due in part to the lack of reliable biomarkers to diagnose the disease in its early stages. Approximately 70% of cancer related deaths occur in less developed countries and many of these are caused by viral infections, including Hepatitis B/C virus (HCC) human papilloma

virus (HPV) (cervical cancer), accounting for 20% of cancer deaths (21,22,23,24). In the United States, cancer is the second leading cause of mortality behind heart disease. Men and women are both highly susceptible to cancer with a 43.9 and 38.0% chance of developing cancer respectively and 22.9% and 19.3% of these resulting in mortality respectively (21). It is estimated that 1,658,370 Americans will be diagnosed with cancer and 589,430 of these will result in cancer related deaths (21).

Cancer demographic statistics and risk factors

The incidence of different types of cancers is summarized in Figure 1. Breast cancer occurs most frequently in females whereas prostate cancer is most common in males (21). As people age the likelihood for developing cancer increases, with some cancer probabilities increasing exponentially (21). Indeed, nearly 80% of all diagnosed cancers occur in persons of greater than 55 yrs. of age, for both sexes. Prostate cancer has less than 1% likelihood in males from age 35-44; however, at 55-64 years of age the number of new cases increases nearly 66 fold and the likelihood increases to 33% (25). Some cancers also have higher predispositions with regard to race, for example prostate cancer is more frequently diagnosed in African American males as opposed to Caucasians or men of other races (25). Despite the ostensible evidence that cancer is age related, at least one third of all cancers are preventable (25). The incidence of these cancers are dependent on lifestyle, including diet, smoking/nonsmoking, alcohol and drug use, working environment, and a sedentary life style (25,26).

Many cancers can be prevented or at the very least delayed by regular screenings, healthy diet, which includes high fiber and cruciferous green leafy vegetables, fruits, low consumption of fried food and processed sugars), and regular physical activity (27). Chronic infections and infections inducing pro-inflammatory cytokines (i.e. high fat diet), some environmental contaminants (polycyclic aromatic hydrocarbons (PAHs), nitrosamines) including carcinogens (benzo [a] pyrene, acridine dyes, nitrous acid asbestos, etc.) also contribute to the increased risk of cancer (27,28). These risk factors can be mitigated by deliberate lifestyle changes and increased awareness of their presence in the everyday environment (28). Being proactive in reducing cancer risk and advocating a cancer preventive lifestyle is the best defense against cancer and has more favorable outcomes than some cancer treatments (28). Genetically-related and other unavoidable cancers are insidious and require development of improved treatment regimens that are less toxic and more patient specific (28,29). This can only be accomplished by a greater and more comprehensive knowledge of the molecular biology of cancer and individual tumors with increasing reliance on a personalized medicine approach (29).

Multistep carcinogenesis

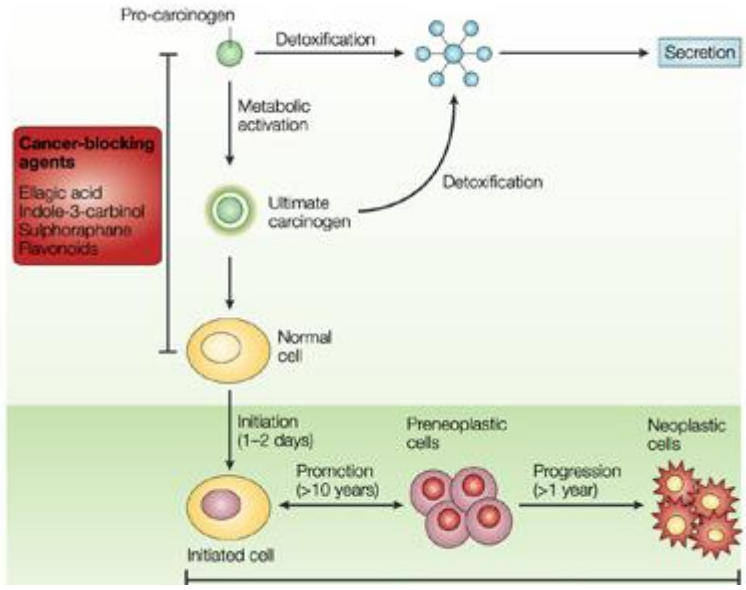

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Figure 1: Model of carcinogenesis. Initiation is started by a carcinogen such as DMBA or benzo[a]pyrene. This damage if unrepaired leads to initiated cells which can be promoted by agents like PKC induced TPA. This leads to proliferation of these initated cell population forming benign neoplasms. Cells then become invasive by loss of heterozygosity (LOH) of key tumor suppressor genes and/or undergoing EMT and overexpressing proteases to digest extracellular matrix (ECM). This is the final stage which is progression and ultimately metastasis.

Initiation

Carcinogenesis (or tumorigenesis) refers to the multistep transformation of normal cells and tissue into a benign neoplasm and then into a malignant aggressive mass known as cancer (Figure 1). Tumor transformation is a multifactorial process and in many models, it can be generalized into three stages: cancer initiation, cancer promotion, and cancer progression (30). Cancer initiation is the first step of carcinogenesis and entails a genetic change induced by a

carcinogen (30). A carcinogen is a type of mutagen, which can be any chemical, or ionizing radiation that induce genetic mutations, which can ultimately lead to tumor formation (27). These species can induce genetic mutations by directly binding, intercalating to or covalently reacting with DNA, preventing the DNA from being properly transcribed into mRNA by RNA polymerase II (RNA pol II) and/or preventing proper replication of DNA (31). It is a generally accepted by regulatory agencies carcinogens that such as benzo [a] pyrene and high energy ionizing radiation, may have no thresholds in their ability to initiate tumors (32). Some chemicals are not carcinogenic at low concentrations by themselves but can initiate or promote carcinogenesis when their exposure is combinatorial with other chemicals or radiation. An example of this is sodium arsenite, which by itself does not cause tumors but does exacerbate tumor size and formation rate of UV-induced tumor formation in mice. Oncoviruses (i.e. retroviruses) can initiate carcinogenesis by integrating their viral oncogenes into the host genome (33). They can also make proto-oncogenes become oncogenes within the host genome by enhancing their expression (i.e. c-Myc, HCV-induced liver cancer) or they can attenuate tumor suppressors (i.e. HPV induced cervical cancer interfering with p53) (33,34). Carcinogens cause mutations and epimutations and these can accumulate as normal cells become transformed malignant cells (35). Carcinogens induce cellular and DNA damage, which provides certain cells with a selective advantage to override mechanisms that have evolved to suppress tumor cell formation (36). In carcinogenesis, there is an aberration in the fine-

tuned balance between cell proliferation and programmed cell death (apoptosis) which results in aberrant cell proliferation and growth. These involve mechanisms that hyperactivate oncogenes and/or inactivate/attenuate tumor suppressor gene function or expression and these are integral components of carcinogen induced initiation. There are many *in vivo* cancer initiation models including one in mouse skin where multiple papillomas form. Topical administration of a carcinogen [i.e. dimethylbenzanthracene (DMBA)] results in the formation of covalent DMBA-DNA adducts (i.e. N7 methyl guanine adducts) which can results in mutations of oncogenes such as H-Ras leading to formation of benign papillomas and eventually squamous cell carcinoma (SCC) (37). However, in this model exposure to carcinogens does not directly result in cancer since most DNA damage is efficiently repaired by DNA repair systems, such as nucleotide excision repair (NER), mismatch repair (MMR), and base excision repair (BER). The former two systems deal with repairing DNA adducts. NER deals with smaller bulky lesions on DNA and uses enzymes such as excision repair cross-complementation group (ERCC 1, 4) Cockayne syndrome type A (CSA, ERCC6), Cockayne syndrome type B (CSB,ERCC8) and DNA repair protein Xeroderma Pigmentosum complementing (XPA, XPB, XPC, XPD, XPE, XPF, and XPG), which remove the lesion and through DNA polymeraseβ resynthesize the excised DNA. BER removes less bulky non helix-distorting lesions, which result in generation of apurinic or apyrimidinic (AP) sites (38). Bulkier lesions require the MMR system and use the enzymes human MutL homolog 1 (hMlh1) and two

(human postmeiotic segregation increased 1and 2 (hPms1and 2)), human MutS homolog 2/6/3 (hMsh2/6/3), and other repair proteins (38,39). These repair systems are very critical to DNA replication and normal cell proliferation. If the damage to the DNA is too great then mechanisms within the cell (p53) can initiate cell death through apoptosis. This can be initiated either extrinsically (i.e. TNFR trimerization and formation of the DISC) or intrinsically (Bax/Bak homo-oligomerization leading to mitochondrial out membrane permeabilization (MOMP)) followed by activation of cysteine-aspartic acid proteases (caspases) that cleave proteins (i.e. PARP, ICAD) and induce cell death (40). These pathways are crucial for normal physiology and it is believed that defects in genes involved in DNA synthesis and DNA damage assessment and repair (during cell cycle checkpoints or apoptosis) can amplify the mutation rate of these initiated cells and therefore enhance tumor formation. These topics will be covered in depth in the “Hallmarks of Cancer” section.

Promotion

Cancer promotion is the second stage of carcinogenesis and is characterized by the clonal expansion of initiated cells. Initiated cells often express aberrant tumor suppressor genes or genes that exhibit decreased expression or are epigenetically silenced. Retinoblastoma (Rb) the heralded guardian of the genome p53, phosphatase tensin homologue deleted on chromosome 10 (PTEN), adenamatosis polyposis coli (APC) are examples of key tumor suppressor genes which are important for cell cycle arrest after DNA

damage (41). Initiated cells, which are refractory to growth inhibitory signals or apoptosis, have increased likelihood to survive allowing them to proliferate and expand. Although carcinogens are no longer required at the promotion stage of carcinogenesis, chronic inflammatory conditions are important for stimulating clonal expansion of precancerous cells (42). Inflammation triggers cells to produce growth factors such as the epidermal growth factor (EGF) which activates mitotic pathways and kinases such as mitogen activated protein kinases (MAPKs) (43). Inflammation also enhances the secretion of the proliferative cytokines such as tumor necrosis factor alpha (TNFα) and chemokines, which attract pro-inflammatory cells such as neutrophils (44). TNFα has a dichotomous role with respect to cell physiology since it stimulates both cell death (hence its namesake) and also cell proliferation (40).

Cell death is triggered by TNFα binding to TNFR (tumor necrosis factor receptor), which trimerizes and recruits TRADD (Tumor necrosis factor receptor associated death domain) by binding to its respective death domains on their cytosolic tails and this complex recruits FADD (Fas associated death domain) (40,45). Fas (CD95) is another death receptor involved in extrinsic apoptosis and through association with its respective ligand, FasL can stimulate apoptosis (45). This will be further discussed in the “hallmarks of Cancer” section under “resisting cell death”. TNFRs recruit TRAFs (TNF receptor associated factor), specifically TRAF2 which also associates with kinase RIPK1, phosphorylating MAP3K14 (NIK NFκB inducing kinase), and this is essential for NFκB activation. NIK

phosphorylates NEMO, which is the regulatory subunit of Inhibitor of κB kinase (46).

These promoted cells can also release reactive oxygen species (ROS) that exacerbate inflammation that can cause DNA damage (47). Hormones can also promote initiated cancer cells to expand by activating hormone receptors. 17β-Estradiol (E2) binds to the estrogen receptor (ERα is the isoform most implicated in carcinogenesis) and upregulate genes required for cell proliferation (48). E2 can also stimulate genes required for angiogenesis inducing expression of vascular endothelial growth factor (VEGF) (49). Moreover, some non or weakly carcinogenic compounds such as phorbol esters (i.e. TPA through PKC), phenobarbital (a classic cytochrome P450 (CYP) inducer) and chlorinated biphenyls (PCBs) can serve as tumor promoters by activating proliferative pathways and stimulating inflammation (50). Inflammation, hormones, and tumor promoters all enhance cell proliferation and contribute to an increase in genetic instability, which drives promoted cells to a malignant state.

Progression

Cancer progression is the third stage of carcinogenesis in which benign neoplastic cells are transformed into malignant cells that exhibit several phenotypic changes including increased growth, dysplasia to anaplasia, loss of polarity, refractory to inhibitory signals from the outside whether exocrine or paracrine, morphological changes, induction of angiogenesis, and invasiveness

(Figure 2). The phenotypic changes in cancer cells are due to irreversible genomic alterations, which include mutations and genetic polymorphisms that accumulate and are amplified in the first two stages of carcinogenesis as well as acquired chromosomal abnormalities such as aneuploidy (51). Aneuploidy includes the acquisition, loss, truncation, or imbalance of chromosomes, which are common karyotypic observations in many advanced cancer cells, especially in cancers of the skin and the colon. The degree of aneuploidy, which may include the loss of either chromosome 8p or 18q or both, is negatively correlated with the 5-year disease-free survival rate in colon cancer patients without metastasis. All these genetic changes in cancer progression advance cells towards a malignant phenotype in which cells are able to invade adjacent tissues and metastasize to non-adjacent organs.

Epithelial to mesenchymal transition

A crucial stage in cancer cell malignancy and invasive propensity is the acquisition of a mesenchymal phenotype by undergoing what is called an epithelial to mesenchymal transition (EMT). EMT constitutes a series of physiological and molecular changes that occur in order for a cell to become a motile, less differentiated, depolarized cell that can detach from the basement membrane and adjacent epithelial cells so they can intravasate neighboring blood vessels and colonize at sites on distal organs (52). These changes all arise by molecular changes in transcriptional machinery and molecular biological composition. Potent transcription factors of EMT such as Snail, Slug, ZEB, Twist,

and Goosecoid upregulate genes associated with a mesenchymal state such as N-cadherin and vimentin and downregulate epithelial associated genes such as E-cadherin. Cadherins are a class of calcium-dependent adhesion molecules that play a role in cell adhesion, polarity and motility (53). In an epithelial cell, E-cadherin associates with tight junctions along with spectrins and α-catenin. In a mesenchymal state, E-cadherin expression is robustly downregulated and N-cadherin expression becomes elevated. Cells also lose sense of apical-basal or up-down polarity and junctions associated with the basement membrane and adjacent cells are lost (54). Epithelial cells that do not undergo EMT can also co-migrate with these transformed mesenchymal cells and have a higher likelihood of establishing a metastatic site in distal organs. This is due to the fact epithelial cells have a greater probability of attaching to the endothelial layer of blood vessels and extravasating into a secondary site (55). Mesenchymal cells that are able to extravasate into a secondary tissue also need to revert from the mesenchymal state back into the epithelial state by undergoing mesenchymal to epithelial transition or MET.

Cancer cell invasion

EMT increases the propensity for cancer cell invasion; moreover, cells acquiring the ability to intravasate blood vessels to colonize at distal tissues, or metastasize is important components and risk factors for decreased patient survival. Indeed, the primary tumor rarely leads to patient mortality but the metastases are frequently lethal (56). Another fundamental characteristic of

malignant cancer cells is their ability to invade the surrounding tissue by upregulation of matrix metalloproteinases (MMPs), motility by formation of invasive lamellipodia and intravasation of blood vessels to colonize to distal tissues. Tumor metastasis represents a highly advanced phase of the disease. Malignant tumor cells can proteolytically degrade the encapsulated boundary, the basement membrane and the extracellular matrix surrounding the primary organ by using proteolytic enzymes such as MMPs, A disintegrin and matrix metalloproteinases (ADAMs), and other proteases, which proteolytically digest the matrix and basal membrane and enable the cells to intravasate a nearby blood vessel or infiltrate a surrounding tissue (56). Proteases such as urokinase also participate in intravasation and lymphatic vessel infiltration.

The process of invasion and metastasis results in tumor formation at a secondary site with tumors that originate from one organ preferentially metastasizing to other organs. For example, tumors that originate from the colon have a higher preference to metastasize in the liver by intravasating the portal vein and extravasating in the liver (57). These primary colon tumor cells can also metastasize to the lung. Indeed, Stephen Paget was the first person to discover the non-random metastatic pattern of tumors after examining over 700 postmortem breast cancer cases, which lead to him proposing the “seed and soil” theory, which suggested that the dynamic interaction between metastatic cancer cells and its respective microenvironment determines the organ preference patterns of cancer metastases. The most frequently targeted organs are the liver,

lung and bone. This directional dissemination is orchestrated by distinctive chemokines in cancer cell chemotaxis, which is an integral component of cancer cell invasion, intravasation, and subsequent colonization at distal organs and tissues (58).

Normal cells undergo extensive changes and acquire distinctive genetic and epigenetic changes, as well as alterations in post-translational regulation and machinery, that are required for the transformed, and the malignant cancer cell phenotype (59). As stated in a famous paper by Weinberg and Hanahan, describes distinctive changes, or hallmarks, of cancer, namely: sustained proliferative signaling, evading growth suppressors, evading cell death, enabling replicative immortality, evasion of immunosurveilance, genomic instability, tumor promoting inflammation, aberrant and deregulated metabolism, inducing angiogenesis, and activating invasion and metastasis (60). Not all of these traits are present in the transformed phenotype or required for cancer cell initiation, promotion, progression and metastasis. However, more often than not many of these hallmarks are acquired sequentially and selected for transformed cells in the most aggressive cancers and the ones most refractory to available chemotherapy, surgery, and treatment.

Hallmarks of cancer: preview

Weinberg and Hanahan published a famous and highly cited paper in 2000 that delineated the complex nature of cancer into tangible underlying principles or

"hallmarks", which has been updated to include new hallmarks in 2011 (Figure 2) (60). Virtually all cancers possess many, if not all, these hallmarks and this has significantly enhanced the way we perceive cancer. Perhaps the most common and rudimentary characteristic of cancer is uncontrolled cell proliferation. Normal cells divide under strict regulation by highly sophisticated and evolutionarily conserved machinery that makes cells pass certain checkpoints within the cell cycle before they can be cleared to proceed into successive steps. The first step in the transformation of normal cells into becoming cancerous is usually a gain of function mutation that involves mutation of a proto-oncogene into an oncogene (60).

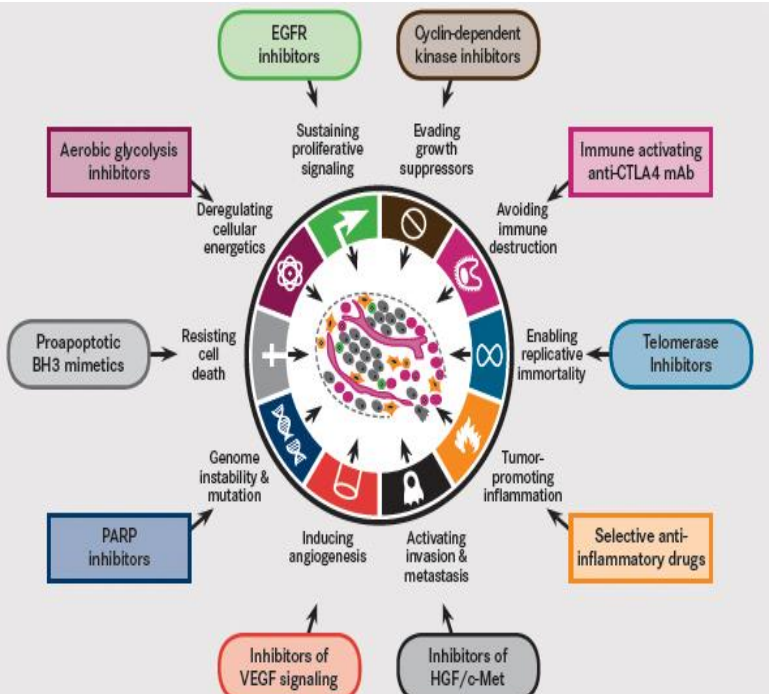

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Figure 2: Hallmarks of cancer. Cancers acquires some, if not all, of these characteristics in a sequential fashion during their progression into a transformed state.

This mutation can make the protein constitutively active and not require the normal activating posttranslational modifications such as kinase-dependent phosphorylations which activate many a receptor tyrosine kinases (RTK) (e.g. Epidermal Growth Factor Receptor (EGFR)) (60). K-ras is an excellent example of such a mutation and is present in at least 33% of all cancers. K-ras mutations are often required in colorectal cancer (CRC) and is listed on the "vogelgram" of CRC progression (61). A missense mutation of K-ras is acquired in amino acid positions 12, 13, or 61 where the corresponding glycine is replaced with a valine, cysteine, or aspartate, resulting in constitutively active K-ras (61). Ras is a small monomeric GTPase and a member of the eponymous Ras superfamily. Its association with guanosine triphosphate (GTP) or guanosine diphosphate (GDP) respectively determines the activation or deactivation of Ras (62,63). Two effector proteins regulate Ras: a GAP (GTPase activating protein) and a GEF (guanine nucleotide exchange factor). Ras has intrinsic, yet very weak GTPase activity, which requires its GAP to fully activate its own GTPase activity (62). When Ras is associated with GAP it hydrolyzes the bound GTP into GDP, making Ras inactive. However, in order to activate Ras the GDP can be exchanged with a GTP by GEF, therefore activating Ras. However, when Ras is mutated at the above-mentioned codons, Ras becomes insensitive to this level of regulation and remains constitutively activated (62,63). Mutation of many other genes such as c-Myc can also lead to uncontrolled cell proliferation. The other hallmarks of cancer include evading growth suppressors and sustained growth/proliferative

signaling (autocrine signaling), deregulated cellular energetics, resisting cell death/evading apoptosis, immunoevasion, inducing angiogenesis, genomic instability (mutations/ translocations, acquiring replicative immortality (telomerase), pro-inflammatory signaling, and acquiring invasion/migratory and ultimately metastatic propensity (60). These hallmarks are acquired through a series of mutations that accumulate over a lifetime and is the result of one's environment, spontaneous mutations, and internal factors pertaining to hormones and other metabolites. In normal cells, there is a balance of factors that are competing with one another to influence the fate of the cell, which can broadly be classified into tumor suppressor genes and oncogenes (64). As mentioned earlier, oncogenes are mutated proto-oncogenes that gain aberrant activity and lose sensitivity to regulation. These kind of mutations are gain of function mutations and lead to aberrant and excessive cell proliferation, growth, migration, invasion and ultimately metastasis (65). Even though cancer is normally associated with these type of mutations and excessive activating of these kind of genes, cancer is largely a "loss of function" disease where critical tumor suppressor gene function is mitigated or completely lost. This can be due to mutations that hinder or abrogate the tumor suppressor function of the gene (hypomorphic, amorphic, or antimorphic (dominant negative) mutations) or loss of gene function due to epigenetic changes that silence expression of the gene [(e.g. hypermethylation of the promoter, histone deacetylase (HDAC)] (65,66,67). Perhaps the most famous tumor suppressor gene is p53, which is heralded as the

guardian of the genome and is often mutated in cancer. Other common tumor suppressor genes that are mutated or epigenetically silenced are APC (CRC), BRCA1/2 (breast), Smad4 (pancreatic, CRC), TGFBRII (CRC), Rb, VHL (kidney), and PTEN (68-71). The alterations in both oncogenes and tumor suppressor genes leads to the transformation of normal cells into cancerous cells in a multifactorial and multivalent successive process called carcinogenesis. This will be described in further detail in the succeeding sections.

The Hallmarks of Cancer

Sustained proliferative signaling

RTKs and MAPKs

The most fundamental and rudimentary characteristic of all cancer cells is their ability to acquire sustained cell proliferation. This is almost universally the first step in becoming a transformed cell and involves contiguous signaling through one or multiple signaling pathways such as mitogen activate protein kinase (MAPK) pathways (72). This represents a vast superfamily of kinases that activate proliferation in response to receptor tyrosine kinase (RTKs) such as epidermal growth factor receptor (EGFR), insulin receptor (IR), and insulin-like growth factor receptor type I (IGF1R) (73). These RTKs are activated by ligand binding on the extracellular side of the membrane, which promotes dimerization of the receptor. Dimerization (either homo- or heterodimerization) promotes

autophosphorylation or “phosphorylation in trans” of the cytoplasmic tails of these receptors on multiple tyrosine residues (hence the name RTK) (74). These phosphorylations create multiple docking sites for proteins that contain src homology domains, specifically SH2 (src homology 2 domain) which recognizes and binds to phosphotyrosine residues (75). Proteins such as Grb2 (growth receptor binding 2) and Shc (src homology collagen) can bind by virtues of these domains and serve as docking proteins for protein kinase cascades (75).

Ras and PI3K/Akt pathway

The Ras protein as stated earlier is a small monomeric GTPase that is activated and repressed by guanine nucleotide exchange factors (GEF) and GTPase activating proteins (GAP) respectively. The known GEF son of sevenless (Sos) binds to Grb2 and activates farnesylation tethered Ras by exchanging GDP for GTP (76). This activates the Ras-Raf-MEK-ERK signaling cascade. These RTKs can also activate the phosphatidylinositol 3, 4, 5-triphosphate kinase (PI3K) pathway. This kinase can bind to RTKs by virtue of Shc/Grb2/GAB interaction or by binding to insulin receptor substrates (IRS1 and 2) (77). Constitutively active Ras is found overexpressed in 30% of all cancers (predominantly K-ras mutations) especially in pancreatic and colorectal cancers. Ras downstream effectors include the pathway of Raf-MEK-ERK. Raf contains three isoforms Raf-1 B-Raf and C-Raf. B-Raf is often mutated to become constitutively active especially in melanoma and drugs that target B-raf such as vemurafinib and dabrafinib can attenuate progression of these B-Raf mutated cancers (78).

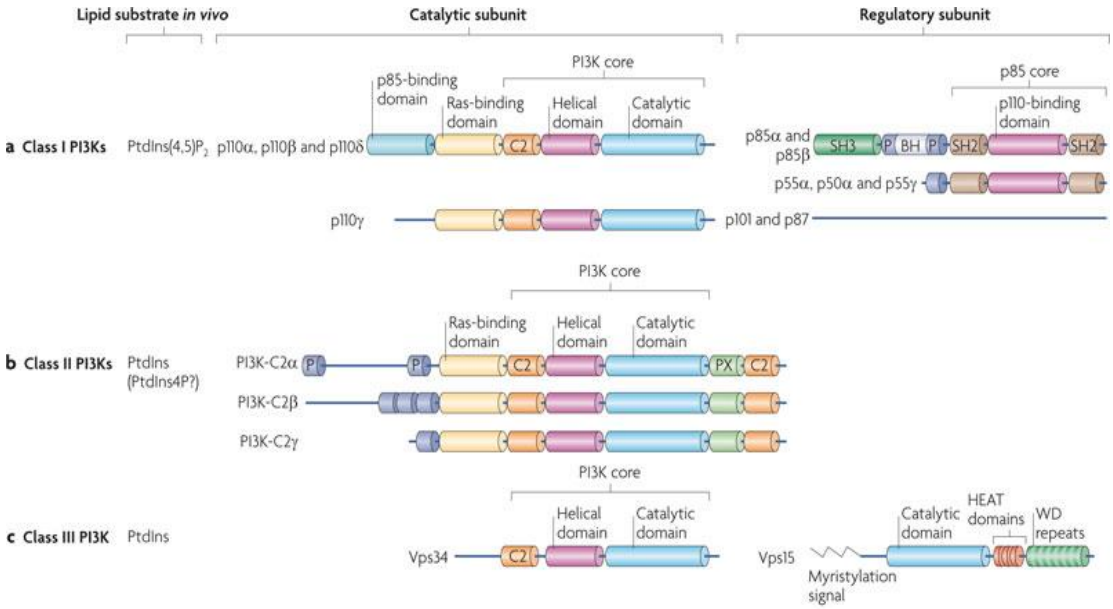

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Figure 3: The three classes of PI3K. They share a high degree of homology and also have significant differences. Class I PI3K family members contain regulatory domains whereas class II lack these. Regulatory domains contain SH3 and SH2 domains (with exception of p87 andp101) which bind to poly-proline and phosphotyrosine domains respectively. Class III has only two members Vps34 and Vps15 (vacuole protein sorting 34 and 15 respectively), which are critical to autophagy. Vps contains a HEAT (Huntington, elongation factor 3 (EF3), phosphatase 2A, and yeast kinase TOR1) and WD domains that are critical for protein-protein interaction. The former motif is found in the mammalian homolog of TOR1, mTOR (mechanistic target of rapamycin), a negative regulator of autophagy. WD repeats (which end in tryptophan (W)-aspartate (D) dipeptides) form solenoid structure domains which facilitate protein-protein interactions

PI3K has three classes: Class I, ClassII, and ClassIII (Figure 3) (79). The first class is the canonical class most implicated in cancer and is composed of two subunits a p110 catalytic subunit and a p85 catalytic subunit (79,80). The catalytic subunit consists of three isoforms: α, β, γ with the first two being ubiquitously expressed and the latter being expressed exclusively in leukocytes, playing a crucial role in regulating the cytotoxicity in natural killer (NK) lymphocytes (80). The p85 regulatory subunit contains five isoforms: p85α, p85β, p50α, p55α, p55γ. The p85 regulatory subunit regulates the catalytic subunit, masking its kinase

domain and inhibiting its ability to phosphorylate its respective substrate phosphatidyl inositol 3, 4 bisphosphate (PIP2).

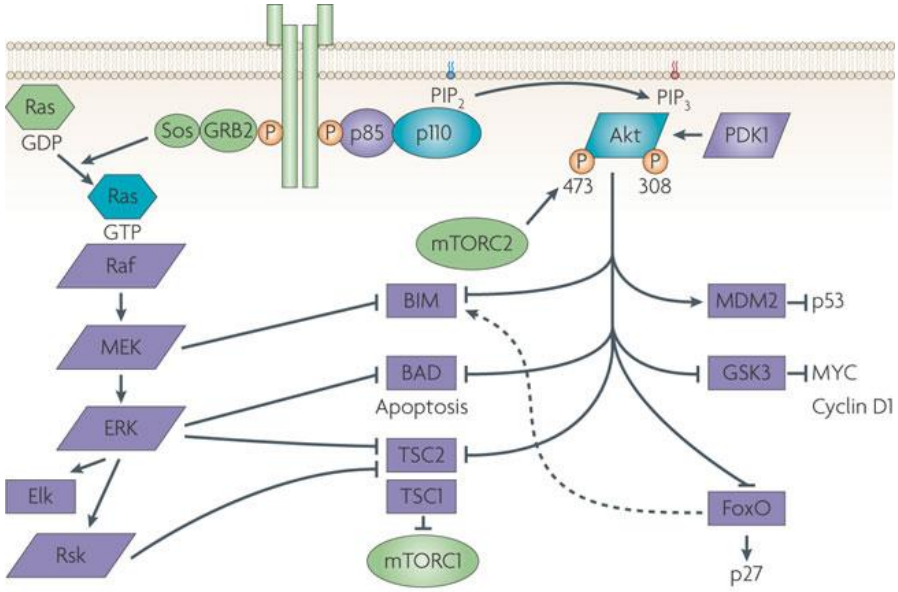

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Figure 4: The PI3K/Akt signaling pathways. Upon receptor activation by ligand binding, PI3K binds to phosphorylated docking proteins (like IRS-1 and IRS-2) and phosphorylates PIP2 (phosphatidyl inositol 3,4 bisphosphate) to PIP3 (phosphatidyl inositol 3,4,5 triphosphate). This phosphorylation is reversible and is catalyzed by the phosphatase PTEN (phosphatase tensin homolog). Formation of PIP3 provides a docking site for Akt by virtue of Akt's Pleckstrin Homology (PH) domain within its N-terminus. Akt is then phosphorylated by PDK1 at T308 and S473 by mTORC2 (or PDK2). Akt promotes cell proliferation and survival by activating MDM2, NFκB, and mTOR (mechanistic target of rapamycin). Akt also inhibits apoptosis by inhibiting caspase cascade, GSK3β (glycogen synthase kinase 3β), and promoting the cytosolic sequestration of FOXO1 (FKHR, Forkhead box protein O1) and Bad bcl2-associated death promoter). The PI3K/Akt pathway is often aberrantly regulated in cancer. This constitutes constitutively active forms of the PI3K catalytic subunit, mutations or epigenetic silencing of PTEN (i.e. Cowden's syndrome), or overexpression of RTKs (such as EGFR or HER2).

Upon receptor activation and association of adaptor proteins, p85 regulatory subunit binds to Grb2/GAB or IRS1/2 and this relieves the catalytic subunit, enabling it to phosphorylate PIP2 to PIP3 (phosphatidyl inositol 3,4,5 triphosphate) (Figure 4). PIP3 serves as a docking site for many proteins and kinases that contain a Pleckstrin Homology (PH) domain (81). One of these

kinases is AKT kinase (also known as PKB) (82). AKT kinase has three known isoforms (AKT 1, 2 and 3 respectively) that are expressed from three different loci on three different chromosomes (82). Upon binding by virtue of its pleckstrin homology domain KT can be phosphorylated and therefore activated on two critical residues: T308 by phosphatidyl inositol dependent kinase 1 (PDK1) and S473 by mechanistic target of rapamycin complex 2 (mTORC2) or PDK2 (82,83). T308 is located within the AGC kinase loop of the protein (83). This motif is shared by other protein kinases such as Protein Kinase A (PKA), Protein Kinase G (PKG) and Protein kinase C (PKC) (hence the name of the loop and the acronym of PKB for Akt) (84). The other phosphorylation site, S473 is located within the hydrophobic pocket of Akt (83). Both phosphorylation sites are associated with activation of AKT; however, phosphorylation of S473 is indispensable for complete activation as it causes a conformational change within the hydrophobic cleft of AKT and consummately activates its kinase activity (85). Akt has multiple downstream targets, activating Tpl2 (86), IKKα (NFκB signaling) (87), PFKB2 (88) glycogen synthase kinase β (inhibitory) (activating glycolysis) (89), AS160 (90), PIP5K (glucose transport) (91), Wee kinase (92), Myt1 (93), p21 (inactivating cell cycle suppressors therefore upregulating cell cycle progression) (92,94). PI3K is often mutated in many cancers, including multiple myeloma, CML, colorectal cancer (95), NHL (96), and breast cancer (metastatic and endocrine resistant and inhibitors such as Idelalisib and duvelisib (PI3Kδ inhibitor, for hematological malignancies) (97), copanlisib (NHL) (96), perifosine (colorectal cancer, although

discontinued since 2013) (95,98), buparlisib (breast cancer) (99), and alpelisib (metastatic breast cancer) (100,101). Akt is associated with multiple cancers, such as colorectal cancer (102), neuroblastoma (103), renal cell carcinoma (RCC) (104), and melanoma (105). Proteus syndrome is associated with a mosaic mutation (E17K), which produces overgrowth of skin, brain, and connective tissues (106). Akt inhibitors such as MK2206 (colorectal cancer) and VQD-002 (neuroblastoma) (107).

mTOR

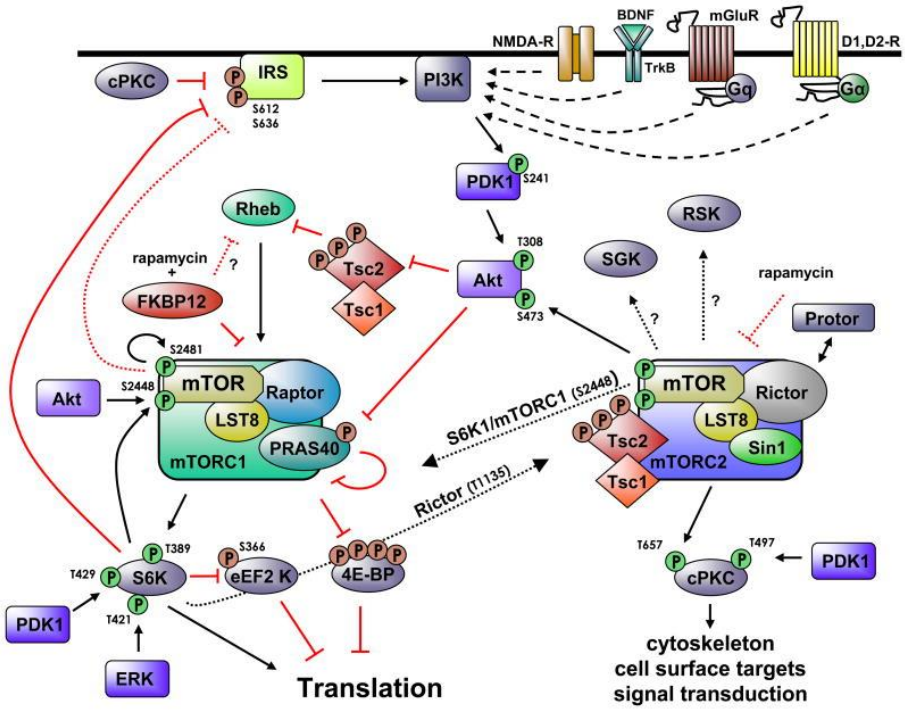

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Figure 5: Mechanistic target of rapamycin (mTOR) signaling pathway. mTOR is positively regulated by rhedb (ras homology enhanced in brain), which is negatively regulated by TSC1/2. mTOR can form two complexes, mTORC1 and mTORC2, which have different components as shown above. mTORC1 regulates protein synthesis by phosphorylating 4EBP1 (4E eukaryotic initiatior factor binding protein) and p70S6K (p70 S6 ribosomal subunit kiase), both of which are critical for ptoein translation.

One of Akt's most integral targets with regards to cell proliferation is mTOR (Figure 5) (82,83,85). The protein mTOR (mechanistic target of rapamycin) is a potent downstream effector of PI3K/AkT signaling and the mammalian/human homolog of the protein TOR found in the yeast *saccharomyces cerevisiae* (82,83,85,108). It is a member of the PI3K-related kinase (PIKK) family, which includes ATM, ATR, DNA dependent protein kinase, SMG1 (nonsense mediated mRNA decay PI3K related kinase), and TRRAP (transformation/transcription domain associated protein) (109,110). These members, along with mTOR have distinct features, which include N-terminal HEAT domains, a FAT (FRAP-ATM-TRAP) domain, a PIKK regulatory domain (which in the case of mTOR is the FRB or FKB12/rapamycin binding domain) a kinase domain, and a C-terminal FATC (FAT at C-terminus) domain (109,110). There are 20 HEAT domains (Huntington, elongation factor 3, subunit of protein phosphatase 2A, and TOR1), each composed of α-helices that mediate protein-protein interactions (109,110). The FAT and FATC domains associate with other factors and aid in activation of mTOR kinase and mutations in either the FAT or FATC inhibits catalytic activity of mTOR (110). The protein mTOR can be a component of two signaling complexes: mTORC1 and mTORC2.

The complex mTORC1 is composed of mTOR, mLST8 (mammalian lethal with Sec-13 protein 8), RAPTOR (regulatory associated protein of mTOR), Deptor (DEP domain TOR binding protein), and PRAS40 (proline rich AkT substrate 40 kDa) (109-111). The components of mTORC2 are mTOR, RICTOR (rapamycin

insensitive component of TOR), mSin1 (mammalian stress activated protein kinase-interacting protein 1), Deptor, and Protor (protein observed with RICTOR) (Figure 5) (109-111). Each of these complexes has its own unique composition and physiological functions. The complex mTORC1 regulates protein translation, ribosomal biogenesis, and promotes an anabolic environment whereas mTORC2 is involved in the regulation of cell survival, proliferation, metabolism, and is a potent activator of AkT (111-113). In cancer, especially RCC mTOR signaling is often dysregulated and has therefore garnered a great deal of interest as a chemotherapeutic target (114-118). The complex mTORC is potently inhibit by the compound rapamycin, an immunosuppressant compound produced by the bacterium *Streptomyces hygroscopicus* on the island of Rapa Nui (Easter Island), where it was first isolated (119). Ever since its discovery a myriad of chemical compounds called rapalogs have been developed in the treatment of cancer, autism, and coronary artery diseases such as atherosclerosis (120-125). Rapamycin and other rapalogs inhibit mTOR by binding to FK506-binding protein (FKB12), which is complexed with rapamycin, then binds with raptor to inhibit its association with mTOR (112,115,120,126). This inhibits mTOR activity; however, it is known to require association with mTOR in order to be catalytically active as knockout of the raptor subunit attenuates mTOR kinase activity, making the entire role of raptor in mTOR equivocal (120,126,127). Raptor is a 150 kDa non-enzymatic component that is essential for mTOR activity and brings mTOR substrates in close proximity (120,126,127). It consists of a highly conserve N-

terminal region with three HEAT repeats and seven WD40 domain containing repeats (domains approximately 40 amino acids in length conserved tryptophan and aspartic acid residues that form anti-parallel beta strands) that mediate protein -protein interacts with mTOR and mTOR substrates (126-128). Raptor is also essential for the formation of the mTORC1 complex itself and mTOR is believed to be complexed with raptor in states of nutrient stress and activation (126-128). Thus, raptor serves as a molecular rheostat of mTOR catalytic activity, and phosphorylation of raptor is an important factor on the dichotomous role raptor plays on mTOR function (127,128). For example, in a nutrient deprived state, AMPK is activated and it phosphorylates raptor on S722/792, and inhibits mTOR activity. Downstream from mTOR, the phosphorylation of raptor by p90 ribosomal S6 kinase (RSKs) on S719, S721, and S863 leads to activation of mTORC1, which is stimulated by EGFR, IR/IGF1R, and other RTK signaling (129,130), which is outlined in figure 5.

PRAS40

PRAS40 (proline-rich Akt substrate of 40kD) is an Akt-substrate and a negative regulator of mTOR (131). Phosphorylation of PRAS on T246 by Akt near its C-terminus leads to dissociation of PRAS40 from mTOR and relieves mTOR of its inhibition (131). PRAS40 contains a putative mTOR signaling motif FVMDE, which is required for the interaction with raptor (131,132). Raptor serves as a scaffold to bring mTOR and PRAS40 in close proximity to promote PRAS binding to mTOR (Figure 5). PRAS40 has also been demonstrated to be a substrate of

mTORC1 at S221 and this phosphorylation seems to be a necessary phosphorylation for the full activation of mTORC1 and in the presence of rapamycin this phosphorylation is abrogated (133). mLSt8 is composed of seven WD40 repeats, which enable binding to the catalytic domain of mTOR and it is hypothesized mLST8 stimulates mTORC1 kinase activity (133,134). Deptor binds to the FAT domain of mTOR and is a component of both the mTORC1 and mTORC2 complexes, with unknown activators or function (132,133,134). AKT also phosphorylates a negative regulator of mTORC1 PRAS40 which inhibits mTOR from associating with raptor which is critical for mTOR kinase activity. MTOR induces cell growth, protein translation, ribosomal biogenesis and proliferation by its two main substrates p70S6 kinase and 4EBP1 (eukaryotic initiation factor 4E binding protein) (134,135). P70S6K phosphorylates S6RP (S6 ribosomal protein) which is a crucial step in the hierarchical assembly of ribosomes (136,137). This also leads to an increase in ribosomal RNA transcription within the nucleolus and its subsequent translocation into the cytosol. Translation is a multistep process that requires the ribosome to recognize specific motifs within the mature mRNA in order to initiate translation (134,135,138). Along with the Kozak sequence, recognition of the 5' 7-methyl guanosine cap is vital for translation initiation (139). Of the many factors involved in translation, initiation eIF4E is vital to recognize the methyl cap; however, when it is in complex with 4EBP1 it is inactivated and translation is substantially halted. Phosphorylation of 4EBP1 by mTOR relieves this association therefore promoting protein translation

(139). Aberrant mTOR activity is associated with many cancers, including breast, prostate, lung, melanoma, bladder, pancreatic, neuroblastoma, glioblastoma, and RCC cancers (140-145). Drugs such as sirolimus (rapamycin), everolimus, temsirolimus, ridaforolimus, and other “rapalogs” are drugs currently in clinical trials (143-146). The drug Tesmirolimus is FDA approved for treatment of RCC (147). mTOR is a critical target for RCC chemotherapy as it is associated with increased HIF1α expression, especially in those patients that have Von Hippel-Lindau syndrome, who are highly susceptible to RCC development (148).

Although mTOR is not a direct substrate for Akt, it is activated by virtue of Akt phosphorylation of Tuberous Sclerosis complex TSC1/2 (149). TSC1 (hamartin) and TSC2 (tuberin) form a heterodimer and serve as a GAP for the monomeric GTPase and one of the activators of mTOR Rheb (Ras homology enhanced in brain) (149). TSC1 and 2 share very little homology with one another and TSC2 GAP domain is activated by association with TSC1, which promotes the hydrolysis of GTP within Rheb to promote its inactive state (150). AKT phosphorylation inactivates this complex by promoting dissociation of TSC1/2 and from Rheb (149,150). There is evidence that Akt phosphorylation also promotes subcellular relocalization of TSC1/2 to the cytosol (as opposed to rough endoplasmic reticulum membrane and/or lysosomal membrane) by promoting association of TSC1 with the sequestration protein 14-3-3 (151). Indeed, many targets of Akt that are negatively regulated by AKT phosphorylation gain affinity for 14-3-3 and are sequestered within the cytosol (149,150). Phosphorylation of

the BH3 only proapoptotic protein Bad inhibits its ability to form pores on the mitochondrial out membrane as it becomes sequestered in the cytosol by this 14-3-3 association event (152). Another proapoptotic protein Bim is regulated in a similar way as well as the potent transcription factor FOXO1 (153).

LKB1 and AMPKα

MTOR can also be negatively regulated by LKB1-AMPKα pathway (154). LKB1 (liver kinase B1; STK11) is a kinase that is part of a trimer that forms AMPKK (AMPK kinase), which includes the pseudokinase activator of LKB1 STRAD (LYK5) and MO25 (CAB39) (155). Endogenous STRAD and LKB1 form a complex in which STRAD activates LKB, which results in a phosphorylation of both partners. MO25 enhances the interaction between STRAD and LKB1 and stimulates LKB1 catalytic activity, serving as a scaffold and regulating the subcellular localization of LKB1 (155). Upon nutrient stress, LKB1 is activated and this leads to phosphorylation of AMPKα (on T172) (156,157). AMPK consists of three subunits (α,β,γ). Each of these subunits plays a role in the stability and activity of AMPK (156,157). The gamma subunit responds to high levels of AMPK (hence the name AMPK) and accomplishes this by binding to AMPK through its CBS (cystathionine beta synthetase) domain and can therefore directly sense the ATP: AMP ratio (158). The gamma subunit contains four CBS domains, which create two binding pockets for AMPK. The binding of AMP to two CBS domains (Bateman domain) causes a conformational change and AMPKγ exhibits cooperative binding activity kinetics. Although AMPK can be activated by AMP

binding, it is relatively weak compared to the phosphorylation of AMPKα (1000 fold) which is a true marker of complete AMPK activation (159). Cancer cells can epigenetically silence AMPK and or suppress its kinase activities by having no expression of LKB1, as seen in HeLa cells (160). However, AMPKα can be activated by p53 or in response to ROS, both which activate sestrin2, which in turn activates AMPK (161,162). This demonstrates a novel way mTOR can be inhibited in both a LKB1 and p53 mutant cancer cell and is chemotherapeutically valuable since ROS inducing anticancer agents can inhibit mTOR in these cancers. Compounds such as doxorubicin, ifosfamide, arsenic trioxide, isothiocyanates, and natural phytochemicals can indirectly inhibit mTOR through this ROS-sestrin 2 mechanism (163-168).

These proliferation pathways are also activated in a ligand independent manner by secreting growth factors that serve as paracrine and autocrine activators of RTKs like EGFR (165). TGFα rests on the outside surface of cancer cells and can be cleaved by intracellular proteases and then activate its receptor EGFR (167,168). These cancer cells can also stimulate and acquire growth factors from adjacent non-tumorigenic cells such as stromal fibroblasts or transformed cancer associated fibroblasts (167,168). Many growth factor receptors are overexpressed or become aberrantly regulated or mutated (hypersensitive to growth stimuli, constitutively active or recalcitrant to growth inhibitory signals). A classic example of receptor overamplification is the HER2 (EGFR2, Erbb2, Neu) receptor in breast cancer (169). This receptor is a member

of the EGFR family and although it has no known endogenous ligand it can heterodimerize rapidly with other EGFR receptors that have bound ligand and overamplify growth signals (169,170). This is an example of oncogene addiction and drugs such as trastuzumab and lapatinib can target such receptors (169,170). Mutated receptors can also amplify a very strong signal in response to very low ligand binding or concentration. In other cases, mutations give rise to hyperactive forms of receptors or downstream effectors of kinase cascades. PI3K can also become constitutively active (i.e. p110αCA) and drugs such as wortmannin, LY294002, 3-methyladenine can inhibit these PI3K driven cancers; however, these drugs are too toxic to be considered for any chemotherapeutic value and are exclusively used in a research based context (171-173). This is because most of these inhibitors are non-specific and inhibit all isoforms and classes of PI3K. Some of these inhibitors are also dual specificity inhibitors and also inhibit proteins such as mTOR. However clinical trials for isoform specific PI3K inhibitors (i.e. p110γ) are being developed (174). In addition, negative feedback mechanisms that normally regulate or counterbalance proliferative are often compromised in cancer cells. The negative regulator of PI3K and its respective phosphatase PTEN becomes epigenetically silenced by promoter hypermethylation in many breast and lung cancer cell tumors (175,176). Excessive proliferative signals also trigger cell senescence, which is a definitive feature or premalignant tumor cells. As cells become more malignant this machinery becomes incapacitated.

Evading growth suppressive signaling

Retinoblastoma (Rb)

Cancer cells must also become refractory to potent growth suppressors that counterbalance the effects of the proliferative signaling. Indeed, late stage cancers frequently represent a loss of function disease as advanced cancers have significantly attenuated, or completely silenced, growth suppressor function. Many growth suppressors are products of tumor suppressor genes, such as Rb and TP53, which negatively regulate cell proliferation by arresting cell cycle progression, activating cell quiescence or senescence, and inducing apoptosis/ cell death (177,178). Functional tumor suppressors are gatekeepers of cell cycle progression and they integrate extra- and intracellular signals. For example, Rb protein transduces inhibitory signals originating extracellularly, whereas p53 integrates intracellular signals that detect cellular stress, nutrient deficiency, malfunctional proteins, and DNA damage (177,178). The primary functions of p53 and Rb are to inhibit G to S phase and serve as sentinels at the G1/S checkpoint. These proteins also regulate the metabolism of dNTPs, glutamine, and glucose, which are necessary for cell growth. Rb is a member of the DREAM complex (dimerization partner, Rb-like, E2F, and multivulval class B) which are genes that participate in cell cycle regulation and repress gene expression (E2F, c-Myc) during quiescence (179,180). Rb normally forms a complex with E2F, a transcription factor that is required for the transcription of proteins known as cyclins (specifically Cyclin E and A) (177,181). Cyclins are vital proteins involved

in the cell cycle and associate with cyclin dependent kinases (CDKs) that fully activate the kinase and participate in the specific stages of cell cycle progression (181,182). The expression of cyclins is very ephemeral (30 mins or less) and as robustly as a specific cyclin expression is induced it is then swiftly degraded and followed by expression of another cyclin (182). The flow of cyclins in cell cycle progression is Cyclin D, Cyclin E, Cyclin A, then Cyclin B, with each having waxing and waning waves of expression (183). As cyclin D expression wanes there is a sequential increase in Cyclin E, then Cyclin A, then Cyclin B (184). When cells are progressing through the cell cycle and CDK4/6 is activated by complexing with Cyclin D, it hyperphosphorylates Rb and this makes RB inactive and unable to bind to E2F. E2F can then activate transcription of Cyclins E and A (185). E2F can also activate PCNA (proliferating cell nuclear antigen) which is the eukaryotic processivity factor required for DNA replication during S phase.

TP53

TP53 (encodes the p53 protein) is the most commonly mutated gene in cancer and due to its very complicated and multivalent role in cell homeostasis, DNA repair, genomic stability, metabolism, and apoptosis (177, 186). This illustrates why p53 is heralded as “the guardian of the genome”. Including the full-length protein p53 encodes 15 different isoforms (186,187). The structure of p53 is modular and it contains an N-terminal acidic transcription-activation domain (TAD) which activates transcription factors such as those involved in proapoptotic activity and a second activation domain (AD2) (187). There is also proline rich

domain that is integral for nuclear exportation by MAPKs and therefore critical for its apoptotic activity, a DNA binding domain (DBD) that binds zinc and contains several arginines, nuclear localization signals, an oligomerization domain (OD) where tetramerization of p53 is required for full activity, and a C-terminal inhibitory domain that suppresses the transcriptional activity of p53 (187). This protein has many cancer inhibitory functions including activation of DNA repair mechanisms. Upon DNA damage, kinases ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) are activated and phosphorylate checkpoint kinases (CHK) 1 and 2. CHK2 is activated by ATM, stabilizes p53, and promotes its activity (188,189). P53 is also a downstream target of ATM and is further activated by decreasing association of p53 with its negative regulator mdm2 (murine double minute 2) which is an ubiquitin ligase that ubiquitinates p53 and promotes its proteasome-dependent degradation (189). P53 activates several genes involved with cell cycle arrest, including p21 (WAF/CIP1), and microRNA (miR) miR34a (190,191).

The pro-apoptotic proteins PUMA and BAX are also upregulated, the latter being a potent apoptotic factor that oligomerizes and forms pores on the mitochondrial outer membrane inducing apoptosis (192). p53 upregulates expression of these genes, and p21 binds CDK to inhibit its ability to promote cell cycle progression (Figure 6). In contrast mutated p53 cannot effectively bind to DNA and induce p53-dependent expression of p21 mRNA (193). Many p53 polymorphisms have been observed and there are mutational "hotspots"

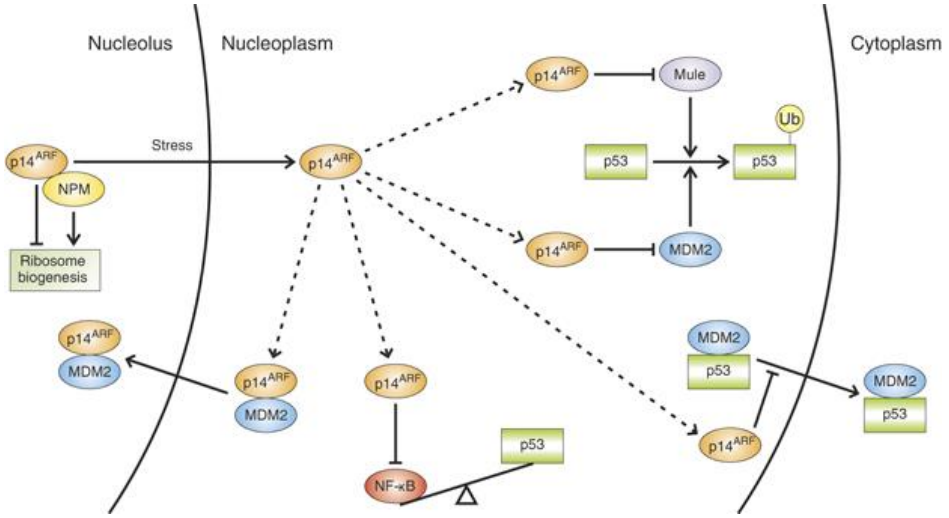

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Figure 6: Rb and p53 pathway. E2F-1 remains in complex with Rb and is inhibited. Upon oncogene activation, such as ras or c-Myc, Rb is hyperphosphorylated, which leads to its dissociated from E2F-1, and degradation of Rb. E2F-1 can then complex with cyclin D1 and transactivate other cyclins such as Cyclin E and other cell cycle genes. The guardian of the genome, p53, negatively regulates the cell cycle, regulates DNA repair, and promotes cell survival. However, under very stressful conditions, p53 promotes apoptosis by upregulating pro-apoptotic proteins such as Bax, NOXA, and Apaf-1. The E3 ubiquitin ligase mdm2 negatively regulates p53 by proteasome-dependent degradation and p53 participates in a feedback inhibition loop by upregulating its own repressor in a metastable state. Mdm2 mediated ubiquitination can be reversed by the ubiquin-specific protease HAUSP (herpesvirus-associated ubiquitin-specific protease).

associated with carcinogenesis: these include R175, G245, R248, R249, R273, and R282 (193,194). These mutations are located within the DNA-binding domain

of p53 and disrupt its conformation and inhibits transcription of p53-dependent tumor suppressor genes. Furthermore, this mutant p53 is a dominant negative protein, squelching WT p53 monomers forming defective tetramers (194). This not only inhibits the tumor suppressor activity of WT p53 but mutant p53 displays some oncogenic functions and transactivates genes such as multiple drug resistance 1 (MDR1), c-Myc, PCNA, interleukin-6 (IL-6), insulin-like growth factor (IGF-1), EGFR, and telomerase reverse transcriptase that are potent pro-oncogenic genes (194-197). MDR-1 is involved in chemotherapeutic resistance, as it is a member of the ATP-binding cassette (ABC) superfamily of pumps involved in pumping out cytotoxic drugs from the intracellular milieu (198). As stated earlier EGFR as well as IGF-1 are involved in excessive cell proliferation and activation of PI3K, Ras/MAPK pathways. PCNA and c-Myc are also drivers of excessive cell growth and proliferation and so is IL-6, the canonical activator of Janus kinases (JAK), which activate STATS (signal transducers and activators of transcription) (199,200). Some STATs are oncogenic transcription factors that upregulate oncogenic genes such as HIF-1α, COX-2, and others (200). Telomerase reverse transcriptase is an enzyme critical for replicative immortality, a hallmark of cancer, which will be discussed later. p53 is negatively regulated by E3 RING (Really Interesting New Gene) finger ubiquitin ligase mdm2 (murine double minute 2) (200,201). The RING finger domain of mdm2 is unique in the fact that it contains a Walker A or P-loop, which confers nucleotide binding and nucleolar localization Mdm2 binds to and blocks the N-terminal trans-activation

domain of p53 and ubiquitinates several lysine residues within the C-terminal domain of p53, leading to proteasome-dependent degradation (201,202). Mdm2 is also a p53-responsive gene that serves as a negative-feedback loop for regulation of p53. P53 protein can undergo proteasome-dependent degradation in an mdm2-independent manner by E3 ubiquitin ligases pirh2 (p53 induced ring H2 domain protein) and COP1 (constitutive photomorphic 1) (203,204). Aberrant regulation and overexpression of mdm2 are associated with cancer and imadazoline drugs, like nutlin, are being investigated as anticancer agents (205).

CDKN2A

CDKN2A is a tumor suppressor gene encoding two proteins that participate in cell cycle arrest and growth suppression: namely, the protein p16 (p16INK4A) and p14 (p14ARF) (206). Both of these gene products function as tumor suppressors in different ways. The p16 protein binds with CDK4/6 to inhibit hyperphophorylation of Rb, therefore leading to the hypophosphorylated active Rb protein (Figure 6) (206). The p14ARF (alternate reading frame) is a shorter gene product because of translation from an alternate reading frame of the *CDKN2A* locus (206,207). P14 is induced in response to elevated mitogenic stimuli (i.e. c-Myc, RAS/MAPK pathway) and accumulates in the nucleolus forming a stable complex with the p53 suppressor mdm2 (206,208). Upon activation by p16 and p14 mdm2 is phosphorylated by these kinases which not only destabilizes mdm2/p53 protein interaction but also induces association with p14 and mdm2, leading to nucleolar sequestration of mdm2 (209).

Desensitisation and inactivation of these growth suppressive signals can also directly lead to loss of apoptotic mechanisms which will be discussed in the next section

Resisting cell death

Perpetual cell proliferation and resistance to growth suppressive signals are characteristics of cells acquiring a more malignant phenotype (210). Normal cells are in equilibrium with multiple signals from the intracellular and extracellular environment that affect cell proliferation and quiescence, survival, and apoptosis (211). Apoptosis is crucial not only for normal tissue homeostasis but also for embryological and limb development. Cancer cells are resistant to apoptosis and other cell death pathways and acquiring these capabilities is a fundamental step in cancer malignancy (212). Apoptosis is a tightly regulated process, which involves activation of proteins known as caspases (cysteine-aspartic acid proteases) that cleave and degrade proteins. This leads to deliberate DNA cleaving, nuclear fragmentation (pyknosis or nuclear shrinkage) followed by karyorrhexis or DNA rupturing, cell blebbing and global mRNA decay (213). This is in sharp contrast to necrosis, which is unregulated, leads to cell swelling, lysosomal swelling, lysosomal and cell leakage, and tissue damage to surrounding cells (214). Not only does this cause damage to tissues but also it is pro-inflammatory and promotes carcinogenesis (214). Apoptosis is activated via intrinsic or extrinsic pathways that are induced within cells by excessive oncogenic signaling, DNA damage, oxidative or nutrient stress and these are all

consequences of hyperproliferation and increased malignant metabolism (aerobic glycolysis) (215). Cancer cells can find loopholes to evade these mechanisms, alter metabolism, increase the flux of glucose, increase expression of antioxidant enzymes (GSTs, glutathione S-transferases) to reduce oxidative stress, and therefore evade apoptotic induction. The two apoptotic pathways are distinct but also converge and lead to caspase cleavage (216).

Extrinsic pathway

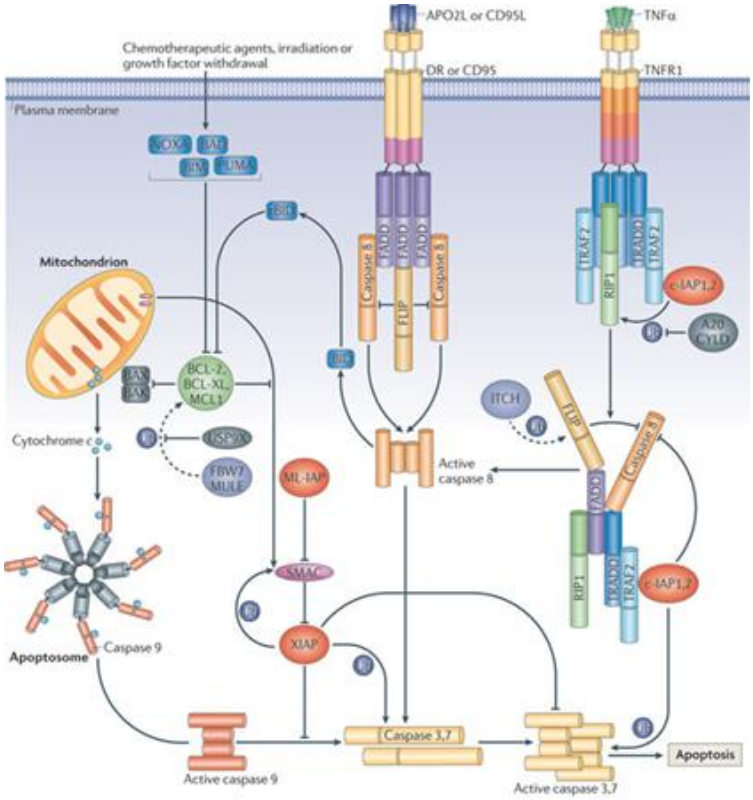

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Figure 7: The extrinsic pathway. This is mediated by TNFα-mediated binding to TNFR (tumor necrosis factor receptor), which trimerize and associate with TRADD (TNFR associated death domain) and enables formation of the DISC (death induced signaling complex). The DISC can also form at Fas/CD95/ FADD (Fas associated death domain) interactions

The extrinsic pathway involves extracellular signaling with Fas ligand binding to Fas (CD95) or TNFα binding to TNFR (tumor necrosis factor receptor) (Figure 7). With TNFα binding to TNFR, the receptor trimerizes and recruits TRADD (Tumor necrosis factor receptor associated death domain) by binding to its respective death domains on their cytosolic tails (217). This kinase exists as a trimer with IKKα and IKKβ serving as the catalytic subunits of the complex where IKKγ (NEMO) serves as the regulatory subunit. TRADD also activates the p38 and c-JNK regulatory cascades.

Likewise, Fas can recruit FADD (Fas associated death domain), which associates with TRADD through their respective death domains and this enables procaspase8 (as well as pocaspase10) to bind by virtue of the caspases DED (death effector domain). This complex is known as the DISC (death induced signaling complex) (Figure 7A) (217). All caspases exist in an inactive state in which the inactive protein needs to be cleaved in order for the caspase to tetramerize and form the active caspase (217). FADD like interleukin β1 converting enzyme (FLICE) induces cleavage of caspase 8 by the DISC and this action is inhibited by cFLIP (cellular FLICE Inhibitory Protein) also known as CFLAR. Caspase 8 (as well as caspase 10 and 9) are initiator caspases (218). When active they cleave effector caspases which cleave proteins (including ICAD or inhibitor of caspase activated DNase) (217,218). Caspases can be divided into two groups: the initiator caspases (2, 8, 9, and 10) and the effector caspases (3, 6, and 7) (218,219). There is also another group of caspases; however, they are

involved in inflammation (caspase 1, 4, 5, 12, 13, 14) (219). These caspases exist in an inactive state and the two families differ with respect to the length of their pro-domains. Initiator caspase contain two domains called a DED (death effector domain) and a caspase recruitment domain (CARD) (220). These domains were initially identified in the *ced-3* and *ced-4* genes in *Caenorhabditis elegans* where many of the component of the apoptotic machinery were first characterized (221). The DED and CARD facilitate association with other proteins that have these domains (221). THE DED is required for assembly with the DISC and other proapoptoic machinery (221). The CARD can bind with proteins that possess a CARD domain including the family of proteins called IAPs (inhibitor of apoptosis protein) (222). Only the initiator caspases contain these CARD and DED domains (222). The caspases contain both a large and small subunit and once the procaspases are cleaved they can tetramerizes in a 2:2 stoichiometry of small to large subunit and become active (222,223). The initiator caspase once activated cleave the effector caspases and irreversibly induce apoptosis. This leads to destruction of ICAD and cleavage of DNA between histones, phosphorylation of the protein H2AXγ and cleavage of the DNA repair enzyme PARP (poly ADP-ribose polymerase) (223,224).

Intrinsic pathway

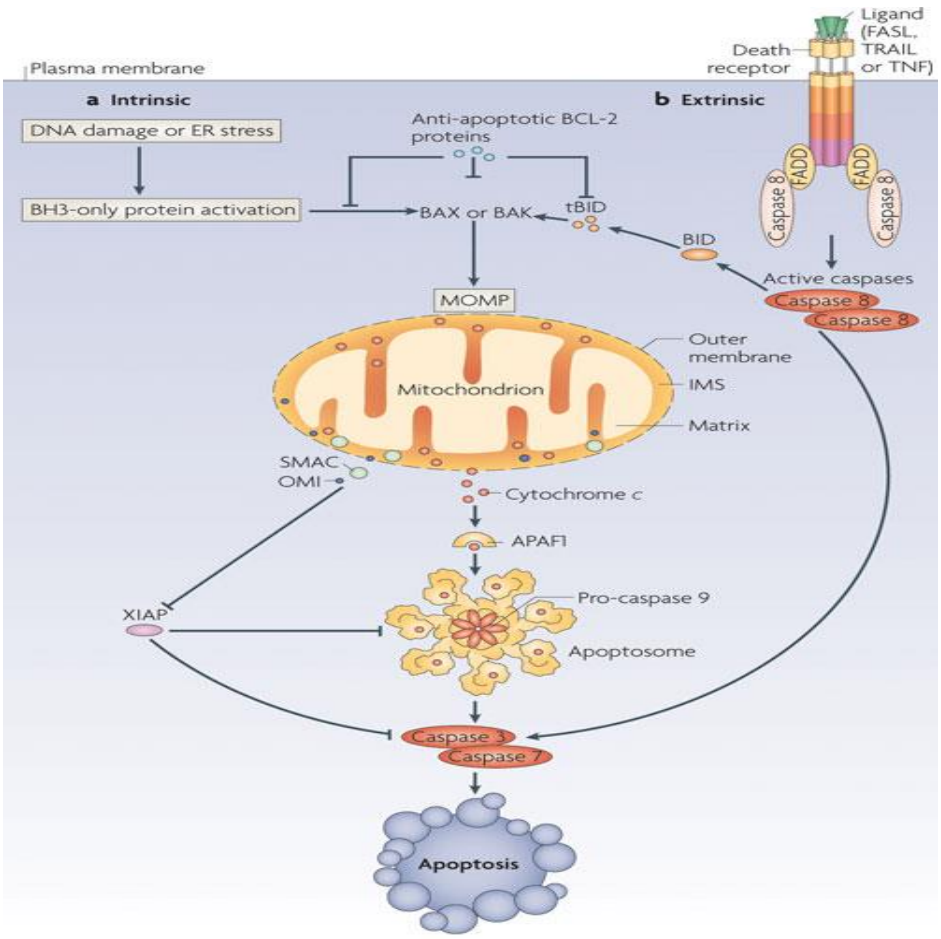

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Figure 8: The intrinsic pathway. Bcl-Xl and bcl-2 form plugs on the mitochondrial outermembrane (MOM) whereas the pro-apoptotic members such as Bax and Bak oligomerizes and form pores, inducing MOM permeabilization (MOMP). The extrinsic pathway can also feed into the intrinsic pathway as the pro-apoptotic member Bid is cleaved (forming truncated or t-bid) and promotes MOMP. Cytochrome c is then released and associates with Apaf1. The inactiave form of caspase 9, procaspase 9, binds with Apaf1 in a 1;1 rato via their CARD domains, forming the apoptosome. This activates effector caspases 3/7 and induces apoptosis.

Along with the extrinsic pathway, apoptosis can also be activated intrinsically. In cancer cells, the extrinsic pathway plays practically no role and is non-functional, and this facilitates the ability to evade immune surveillance. CD95 negative tumors can overexpress FasL, which induces apoptosis in infiltrating T lymphocytes (225). Stimulation of the TNFR by TNFα also promotes NFκB signaling as well as inflammation and release of cancer growth factors (226). The intrinsic pathway is dependent on molecules that interact with and regulate the integrity of the mitochondrial out membrane (MOM) (Figure 8).

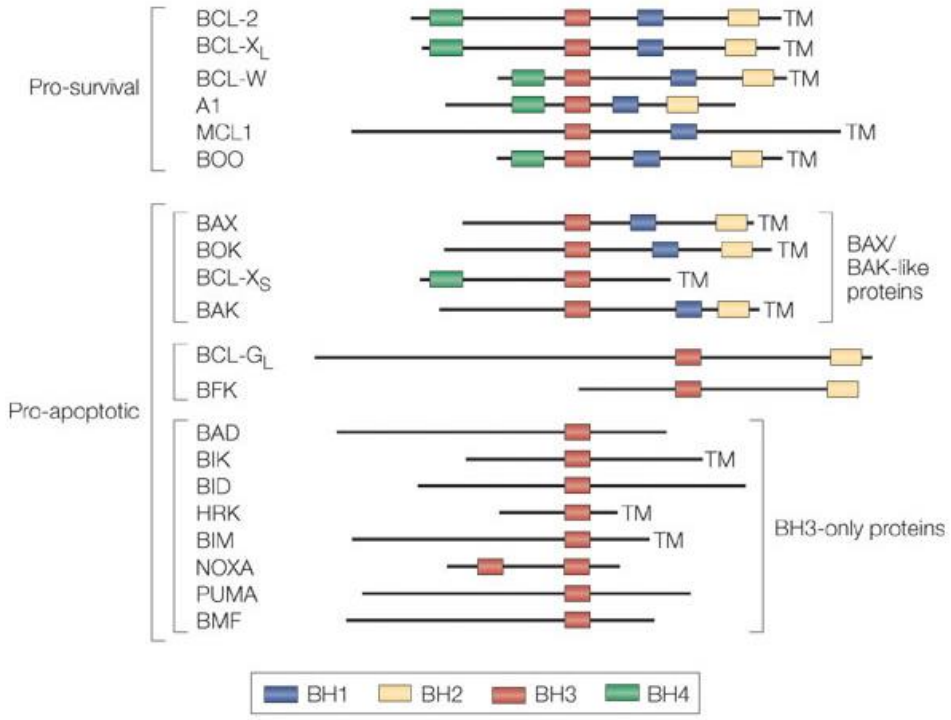

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Figure 9: Bcl-2 family. These members are distinguished by possessing bcl-2 homology (BH) domains. BH1 and BH2 domains are associated with membrane insertion and interaction with the MOM. Pro-apoptitc proteins contain a BH3 domain and lack a BH4 domain. Certain members such as Bim (bcl2-like protein 11), Bik (bcl2-interacting killer) and PUMA (p53 upregulated mediator of apoptosis) possess only a BH3 domain and interact with other bcl-2 family members through their respective BH3 domains. The presence of a BH4 domain confers anti-apoptotic activity.

A large superfamily of bcl-2 homology (BH) proteins that can contain up to four different BH domains (1, 2, 3, and 4 respectively) regulate this pathway (227-229) (Figure 8 and Figure 9). There are also three major classes in this superfamily: antiapoptotic proteins, the pro-apoptotic proteins, and the BH3-only pro-apoptotic proteins (Figure 9) (227-229). The anti-apoptotic proteins are distinguished by possessing a BH4 domain, which confers antiapoptotic activity and this includes bcl-2, bcl-Xl, and Mcl-1 (227-229). These proteins are positioned on the MOM and serve as plugs (forming homo- and heterodimers), preventing mitochondrial outer membrane permeabilization (MOMP), a hallmark feature of apoptosis (227). These proteins interact with one another by associating through the BH regions 1,2, and 3 which is mediated by αhelices 2, 7-8, and 4-6 respectively (229). When bound to pro-apoptotic members, their BH4 domains undergo conformational changes and theses “plugs” become disrupted (227,228). Anti-apoptotic proteins also prevent oligomerization of the pro-apoptotic members and inhibit the activity of VDAC (voltage gated anion channel), a critical member of the mitochondrial permeability transition pore complex (MPTP), required for the release of cytochrome c and Ca^{2+}, which are steps in apoptosis (Figure 8) (228,230-232). The pro-apoptotic members include Bax (Bcl-2 associated member X) and Bak (Bcl-2 homologous antagonist killer). Bax was originally identified by coimmunoprecipitation studies with bcl-2 and Bak was identified and cloned by multiple groups. Both proteins are composed of nine α helices, with a C-terminal transmembrane domain, and an αhelix 9, which is essential for

tethering to the MOM. Bax has high affinity for bcl-2 whereas Bak has higher affinity for Mcl-1, and both have equal affinity for bcl-XL. Bak is constitutively bound to the MOM, whereas Bax is cytosolic and upon apoptotic stimuli, such as p53 activation, Bax localizes to the MOM. Helix 9 of Bak is always exposed whereas helix 9 of Bax in a resting state interacts with its hydrophobic core and needs to be exposed by conformational changes within the protein after apoptotic stimuli. As stated earlier p53 upregulates Bax and activates it. Bax is normally in the cytosol but under apoptotic stimuli it is translocated to the MOM and can interact with and stimulate the opening of VDAC (192,227,228). Bax along with Bak can also form oligomeric pores known as mitochondrial apoptotic channels (MAC) on the MOM (192,226,227,228). This leads to the release of cytochrome c from the mitochondria along with other apoptotic factors (i.e. AIF, SMAC/Diablo) (228,229). Bcl-2, bcl-xL, and also VDAC2 can prevent formation of the MAC by binding to Bak (227-229). The BH3 family members are also pro-apoptotic and are induced by a number of stimuli including heat, mechanical stress, peroxide, and increased acidity (227,228,230). These members have only a BH3 domain as well as a transmembrane domain and include Bad, Bid, Bim, Bix, Blk, and Bnip (227-230). One of these proteins, Bid, can be cleaved by caspase 8, which is activated by the extrinsic apoptotic pathway, and become truncated Bid (tBid), which binds MOM and induces MOMP (230). These proteins along with the other pro-apoptotic bcl-2 family members decrease mitochondrial membrane potential (MMP), leading to cytochrome c release. The release of Apaf1 and cytochrome c

leads to the formation of the apoptosome, a heptameric structure consisting of a 1:1 stoichiometric ratio of cytochrome c, apaf1, active caspase 9 and dATP, which leads to the activation of caspase 3 (230-232).

Bcl-2 family proteins and cancer

Cancers can evade apoptosis by overexpressing antiapoptotic members such as bcl-2 and bcl-xL. Indeed in follicular lymphoma a chromosomal translocation occurs between chromosomes 14 and 18 (t (14:18)) (233). This places the bcl-2 protein-coding region next to the immunoglobulin heavy chain locus on chromosome 14, leading to very high expression levels of bcl-2 (232). The p53 tumor suppressor is also critical for induction of apoptosis, including BAX and transcription of the lincRNA (lincRNA-p21, the antisense transcript of the p21 mRNA) with the latter being required for hnRNP-K induced apoptosis (192,193,227,233). Exploiting the fact that BH3 confers pro-apoptotic propensity, the development and innovation of BH3-mimetics and Bcl-2 inhibitors are being extensively investigated (234). These include drugs like ABT-737, which selectively targets Bcl-2, bcl-xL, and bcl-W and has delivered desirable results in patients with B-cell malignancies and patients with small lung cell carcinoma (SCLC) (235,236). A similar drug ABT-263 has also demonstrated efficacy in SCLC as well as the drug obatoclax (GX15-070) (237). Another drug that has shown promise as a BH3 mimetic is venetoclax (ABT-199) which blocks bcl-2 and favorable responses have been reported (238).

Inhibitors of apoptosis proteins

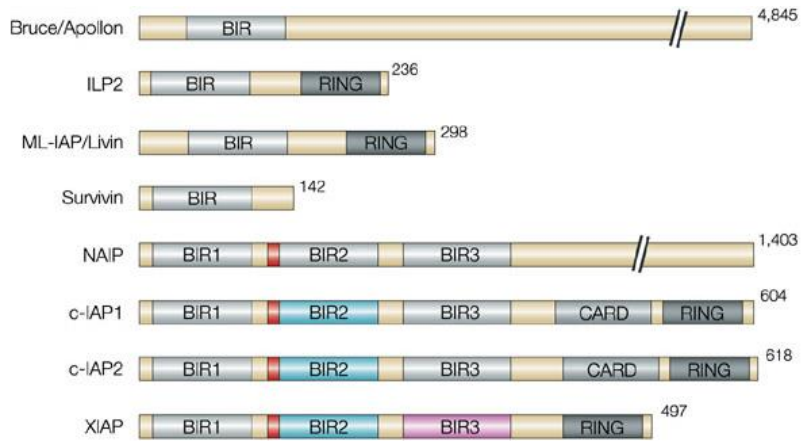

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Figure 10: Inhibitors of apoptosis (IAP) family. They are identified by the presence of BIR (bacuolviral IAP repeats). These proteins inhibit apoptosis by inhibiting the activitaion of procaspases (like surivin), or can directly bind to effector caspases (XIAP). Surivin and XIAP are the two most studied IAP proteins and have the best-documented roles in cancer. These IAPS can also possess RING fingers and can serve as E3 ubiquitin ligases, which can ubiquitinate substrates for proteasome-dependent degradation or auto-ubiquitinate (XIAP). The proteins c-IAP1 andc-IAP2 have a more equivocal role in apoptotis and also participate in cell survival and necroptotsis via TRAF2 and TRAF6.

Cancers also upregulate the class of proteins known as inhibitors of apoptosis (IAP) proteins (239). These proteins were originally identified in the baculovirus, and are important for its reproductive cycle in lepidopteran cells (239, 240). IAP proteins contain eight members (NAIP, cIAP1, cIAP2, XIAP, survivin, Bruce, ML-IAP, and ILP2) which are encoded from the genes *BIRC1-8* respectively (Figure 10) (239). The proteins are so named because they possess baculoviral IAP repeats (BIR) domains, which are involved in anti-apoptotic activity, and the two most well studied and implicated in cancer members, are surivivin and XIAP (241,242). Survivin is the smallest IAP member (being 16-17

kDa) and has only one BIR domain and a coiled coil domain (241). Survivin inhibits activation of procaspase 9 and 10 by virtue of its BIR domain and in many cancers, including colorectal and pancreatic cancers survivin expression is elevated (241,243). Survivin has been a therapeutic target of much interest and its expression is often a negative prognostic factor for cancer patients (241,243,244). Strategies for targeting survivin include drugs that inhibit promoter activity (such as YM155), decrease stability of the mRNA (antisense oligos), inhibit upstream activators (RTK inhibitors, Raf inhibitors, MEK/ERK inhibitors), protein stability and folding [HSP90 inhibitors geldamycin (17-AAG), shepherdin] and its epigenetic regulation (HDAC inhibitors like belinostat), Aurora kinase B/PLK-1 inhibitors) (245-259). The drug YM155 has exhibited therapeutic potential in colorectal cancer, prostate cancer, melanoma as an adjuvant to docetaxel treatment, and in cisplatin-resistant HNSCC (245-248). Other compounds used to inhibit survivin transcription are M4N (Tetra-O-methyl nordihydroguaiaretic acid) by targeting Sp1, a transcription factor important for surviving expression (260). Cotreatment of M4N plus temozolomide (TMZ) enhanced cell death and inhibited proliferation of glioblastoma cells (261). Mithramycin has also been of use as an agent to target survivin by virtue of its inhibition of Sp1 interactions with the survivin promotor (262,263).

XIAP is also a negative prognostic factor for colorectal, pancreatic, prostate, ovarian, lymphomas, AML (acute myelogenous leukemia), and ALL (acute lymphoblastic leukemia) patients (242,264-270). XIAP is the only IAP

member that directly inhibits effector caspases 3 and 7 by binding with the BIR2 domain of XIAP through their IBM (IAP binding motif) (242,264,265). XIAP also inhibits caspase 9 directly by its BIR3 domain (Figure 10) (264,265,269). XIAP also contains an UBA (ubiquitin association domain) as well as cIAP1, cIAP2 and RING finger domains, which is one of two type of domains associated with E3 ubiquitin ligases (the other being HECT type E3 ligases, which are found in proteins such as SMURF) (264-266). There is evidence that in normal or apoptotic conditions, XIAP through its E3 ligase activity can auto-ubiquitinate and promote its own proteasome-dependent degradation (271). In a survival context however, Akt can phosphorylate XIAP on S87, to promote association with survivin and this stabilizes XIAP (271). In apoptotic cells, SMAC (secondary mitochondrial activator of caspases) is released from the mitochondria and through its IBM can bind with XIAP leading to its proteolytic degradation (263-265,272). XIAP is overexpressed in many cancers, and is a negative prognostic factor, and also a potential therapeutic target (264-270). These strategies include antisense morpholinos and oligos that target XIAP mRNA to reduce its expression such as the antisense oligonucleotide AEG35156 developed for treating AML (271). However, success with this strategy has been limited and phase I/II clinical studies have shown severe neurotoxicity (271). A much more viable strategy has been the development of compounds that bind to the BIR3 domain of XIAP, namely mimetics of the protein SMAC/Diablo, and the SMAC mimetic TL32711 exhibited anti-tumor activity in solid tumors and lymphomas (272). SMAC

mimetics GDC-0917 and HGS1029 have also shown promise in Phase 1 studies and antitumorigenic activity in solid tumors and lymphomas, and the mimetic LCL161 from Novartis was effective in TNBC (triple negative breast cancer) (273-275).

Necroptosis

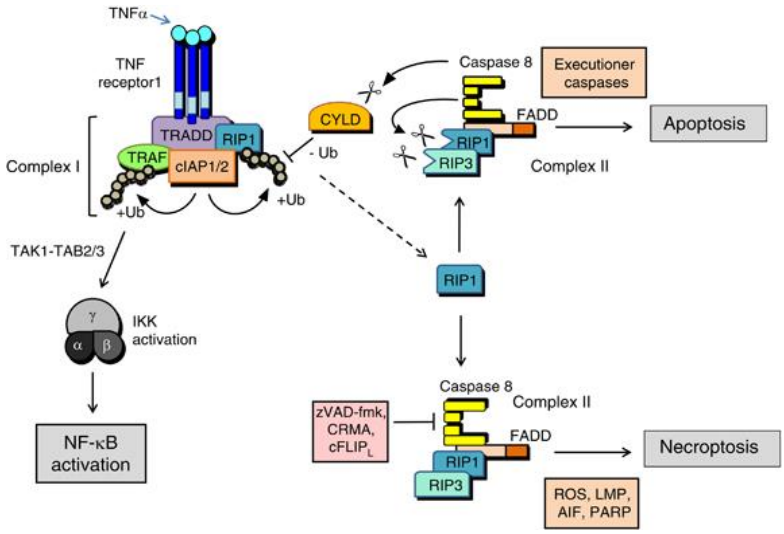

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Figure 11: Necroptosis. Necroptosis is programmed apoptosis that involves the activity of RIP kinases 1 and 3. RP1 binds to FADD and TRADD in response to TNFα which facilitates binding of RIP3. TRAF2,5, and 6 are recruited to the necroptotic complex and their cytosolic tails are ubiquitinated (K63) by cIAP 1 and 2 (cellular IAP proteins 1 and 2). This complex forms along with MLKL, PGAM5L, PGAM55, and DRP1 and induces apoptosis. This pathway is very prevealent in cells where caspases are intactvated even in response to apoptotic stimuli. Caspase inhibitors, like zVAD-fmk also promote this pathway

In addition to apoptosis, another cellular program that results in death is a process called programmed necrosis or necroptosis. This is a mode of programmed cell death that is independent of caspase activation and involves proteins called RIP (receptor-associated protein) kinases (Figure 11) (276). It was originally thought that apoptosis was always favored over necrosis, however in

certain cases, especially with the immune system and pathogen infiltration, a form of regulated necrosis is favorable. This involves RIP1 binding to FADD and TRADD in response to TNFα which facilitates binding of RIP3. RIP1 and 3 recruit TRAF 2, 5 and 6 as well as cIAP 1 and 2 which can ubiquitinate the TRAF tails (on K63) and stimulate downstream necroptotic signaling (277-280). RIP1, RIP3 along with MLKL phosphoglycerol mutase family member 5L (PGAM5L), PGAM55, and dynamin related protein 1 (DRP1) can form the necroptosome, which binds mitochondria to induce mitochondrial fission and necroptosis (276-280). This pathway is especially favored in response to caspase 8 inhibition, which is another evolutionary adaptation for normal cells where caspases are inhibited or mutated. In cancer cells apoptosis is blocked and cells have another way to undergo cell death to prevent tumor formation (280).

Autophagy

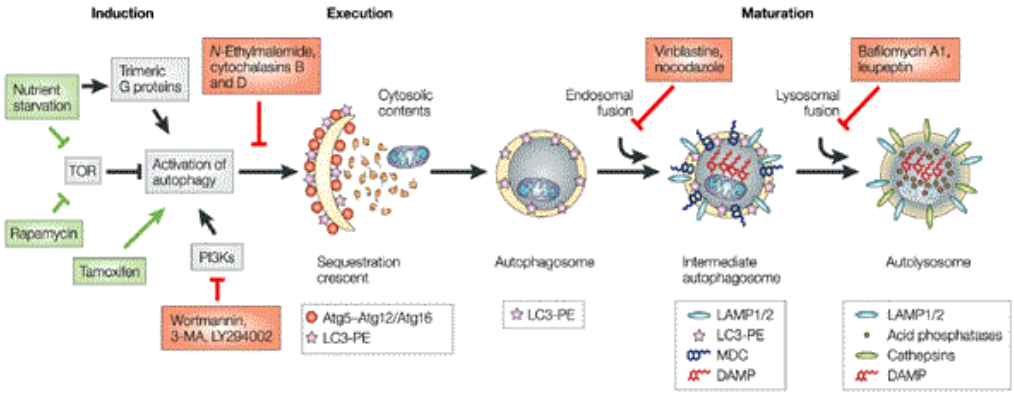

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Figure 12: Classical autophagy pathway. The phagosome forms and elongated by beclin1, ATG14L, Vps15, and Vps34. The contents of the autophagosome then fuse with the lysosme where the contents are degraded. Chloroquine, baflomycin A, and Azithromycin inhibit this interaction. Cancer cells become resistant to many drugs and chemotherapeutic regimens by exploiting this pathway.

Cancer cells can also exploit autophagy as a way to survive in times of nutrient stress, protein instability, and cytotoxicity derived from chemotherapeutic agents (Figure 12) (281). ULK1 is required for the formation of the preautophagosomal structure along with other autophagy related genes (ATGs) 13, 101 and FIP200 (281). After induction and initiation, there is nucleation and elongation of the phagophore until it becomes a phagosome and these steps are regulated by beclin1, ATG14L, Vps15, and Vps34 (vacuole protein sorting proteins, class III PI3K kinases) (282,283). The full closure of the autophagosome requires ATGs 5, 12, and 16 along with insertion of phosphatidyl ethanolamine conjugated LC3 into the autophagosome membrane (281-283). ATG5 and ATG12 are linked together by ATG7 (an E1-like protein) and ATG 10 (E2-like protein) whereas phosphatidyl ethanolamine conjugation of LC3 (LC3-I to LC3-II, which is necessary for autophagasome maturation and fusion of a lysosome) is associate with ATG7 and ATG3 (an E2-like protein) and both these ubiquitination pathways are critical for autophagy (281-283). ATG5 and ATG12 linkage promotes interaction with E3-ligase protein ATG16L promotes LC3I PE (phosphatidylethanolamine) conjugation fusion with a lysosome (forming an autophagolysosome) where the contents are degraded by pH sensitive autosome hydrolases, and recycled for other metabolic processes within the cell (ATG4 removes the phosphatidyl ethanolamine and recycles LC3) (281-284). Proteins such as ESCRTS (endosomal complexes required for transport), SNARES (N-ethylmaleimide sensitivity factor associated receptor proteins) and Rab 7 also

promote this interaction. Other SNARE complexes that are implicated in autophagosome maturation include syntaxin 17 and VAMP 8 (vesicle associated membrane protein 8) by SNAP 29 (synaptosome-associated protein 29) (281-284). This process is negatively regulated by mTOR and positively regulated by AMPK (AMP kinase) (282-285). MTOR phosphorylates ULK1, therefore inhibiting its activity and activation of the PAS (phagophore assembly site). When mTOR is inhibited by AMPK, ULK1 kinase is active and phosphorylates FIP200, ATG13 and itself (282-285). This leads to the maturation of the phagophore, which includes the acquisition of Beclin-1, ATG14L, vps15 and vps34 (284,285). Other positive regulators of phagophore elongation that are independent of mTOR activation include AMBRA1 (autophagy and beclin 1 regulator 1), Bif1 (bax interacting factor 1), and UVRAG (UV radiation resistance-associated gene protein) and negative regulators include Rubicon, Bcl-2, Bcl-xL, and Bim (284-285).

This mechanism is similar to how cancer cells also use the ubiquitin proteasome-dependent pathway to remove potentially toxic, nonfunctional proteins to prevent further protein misfolding, formation of aggregates further oxidative damage, and recycle proteins for amino acids. This has made both autophagy and the proteasome attractive targets for treating some cancers and their roles might not necessarily be mutually exclusive. Indeed, research has shown that in ovarian cancer resistant to cisplatin/carboplatin treatment rely heavily on autophagy to overcome the high amounts of DNA-crosslinking and

oxidative stress and damage. Bortezomib (Velcade) is a well-known 26S proteasome inhibitor that binds to the catalytic site of the proteasome. Bortezomib induces early stage autophagy in carboplatin-resistant ovarian cancer yet inhibits late stage autophagy (286,287). Treatment with bortezomib increased formation of several lysosomal-autophagic vacuoles that accumulated throughout the cell (286,287). This was due to decreased activity of the lysosomal hydrolases cathepsins, and the inability of the cell to break down the contents of the autophagolysosome (286,287). It is known that autophagy can induce apoptosis by the excessive accumulation of autophagic vacuoles (286,287). Cancer cells rely heavily on autophagy in later stages due to the increased demand for building blocks for cell membranes and energy through aerobic glycolysis and the Warburg Effect. Cancer cells are also prone to excessive oxidative damage and autophagy is implemented in order to break down damaged proteins and prevent further damage. There are several stages of apoptosis that can be inhibited and likewise certain stages of autophagy inhibition can result in differential effects. Mechanistically, autophagy and apoptosis are linked and have some interrelated pathways (288). One of the proteins critical for autophagosome maturation is p62, which is also important for activation of NF-κB (by association with TRAF6) and activation of caspase 8. Caspase 8 and p62 share a mutual regulatory role and one can degrade the other (281-285,288). With formation of the death induced signaling complex (DISC), caspase 8 degrades p62, which inhibit later stages of autophagy. On the other hand, in later stages of autophagy it has been shown

that caspase 8 can be degrades by p62-mediated autophagy (281,284. However, p62 needs to be degraded for proper autophagosomal-lysosomal fusion. If caspase 8 is degraded before degrading p62, then formation of autophagosomal vacuoles and puncta can aggregate, inhibit cell function, and promote apoptosis by oxidative damage, excessive aggregation of misfolded proteins, and induce mitochondrial outer membrane permeablization.

When operating at low levels, autophagy is a survival strategy for cancer cells, however when overly exploited cancer cells can become irreversibly dormant and this leads to apoptosis. Drugs such as chloroquine and bafilomycin A have similar effects and inhibit formation of the autophagolysosome (289). Vps34, which is required for nucleation and elongation of the autophagosome, is a PI3K family member and can be blocked by PI3K inhibitors such as wortmannin, 3-methyladenine, LY-294002 (79-81, 283-285). Class 1 PI3K is necessary for activation of AKT, which is vital for mTOR activation and inhibiting PI3K can indirectly induce apoptosis due to a drastic reduction in protein synthesis. Activation of ERK and MEK is also important for autophagy and inhibitors of these respective proteins can have the same effects as PI3K inhibitors. Currently there are Vps34-specific inhibitors available, such as SAR405, which binds to the ATP binding cleft of the protein (79-81, 290). Evidence suggests that this type of inhibitor could serve as a potential anticancer agent when used as an adjuvant in RCC treatment with FDA approved everolimus (291). Excessive self-consumption can lead to cell death as expression of beclin1 in many tumors is

lost. Therefore, autophagy has a dichotomous role in cancer and certain molecules and mechanisms serve as rheostats for regulating the activation and duration of autophagy.

Enabling replicative immortality

Telomerase

Cancer cells can proliferate continuously, however they also face another hurdle: namely, the problem of telomere shortening with each successive cell cycle. DNA is replicated in S phase of the cell cycle and the mechanisms of high fidelity DNA replication and processivity have evolved to pass on the genetic information from one cell to daughter progeny cells effectively and with high fidelity. DNA is replicated by enzymes known as DNA polymerases, which require an RNA primer, since they cannot synthesize DNA *de novo*. In humans, this RNA template is made by the primase DNA polymerase α and then the strand is synthesized by DNA polymerases δ and ε (292-294). RNases then remove the RNA template and DNA polymerase δ resynthesizes the remaining gap, using the DNA 3' as a "primer" to resynthesize the gap (292-294). This is performed on both the leading stand, which is continuous, and the lagging strand, which is discontinuous and has multiple RNA primers and Okazaki fragments (292-294). DNA replication is always in the 5' to 3' direction and the lagging strand is halted at the replication fork, hence the punctuated periods of replication. Eukaryotic DNA is linear and exists within structures called chromosomes. Chromosomes

contain a long q arm and a p short arm, one centromere and two identical pairs of chromatids (294). At the ends of these chromatids are structures called telomeres that are dynamic structures, which constantly interchange between a closed confirmation to block recognition by DNA repair enzymes to an open confirmation during S phase in the cell cycle. During S phase in cell division these telomeres are continuously shortened because when the RNA primer is removed, DNA polymerases have no primer to use to resynthesize the removed RNA primer (292-294). This results in shortened telomeres, leading to chromosome instability, which triggers cellular senescence and death (293-295). In order for cells to become malignant and progress they must acquire replicative immortality and gain unlimited proliferative capacity (296). In 1961, Leonard Hayflick observed that cultured human fetal cells divide only 40 to 60 times and this phenomenon was termed the Hayflick limit (297). A good example of this is the HeLa cell line (cervical cancer cells derived from patient HenriettaLacks) established in 1961 and still used today as a cancer cell model. The molecular basis of this Hayflick limit is the shortening of the telomeres after every successive cell

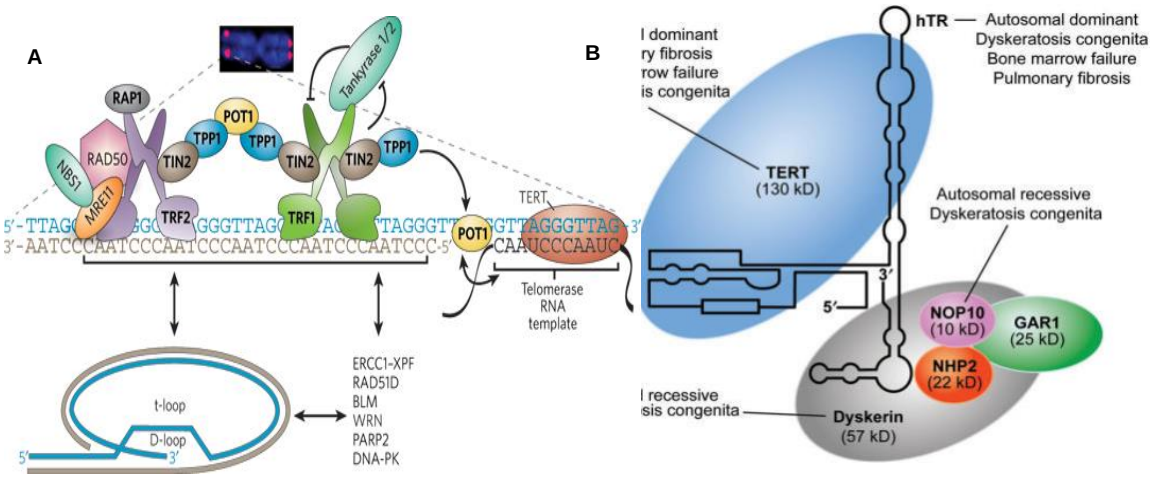

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Figure 13: Telomeric complex and telomerase. A) The structure of telomeric complexes that form to protect telomeres from degradation by DNA repair mechanisms. This includes shelterin (composed of TRF1, TRF2, POT1, tankyrase, TIN2, and TPP1. Tankyrase is a member of the poly-ADP-ribosyltransferase (PAR) family of proteins and mediates length and stability of telomeres by PARsylating TRF1. The proteins RAD50 , 3'-5' endonuclease MRE11, NBS1, and KU70/80 mediate homolgous recombination during telomeric repair. B) Components of telomerase: telomerase reverse transcriptase (TERT), human telomerase RNA component (hTRC or HTR) and dyskerin (which contains ribonucleoproteins NOP10 , GAR1, and H/ACA ribonucleoprotein complex subunit 2 (NHP2)). Telomerase carries its own unique RNA template, which is hTRC, and is approximately 451 nucleotides in length. TERT uses the template region of TERC (3'CAAUCCCAAUC-5') to add TTAGGG repeats to the 3' end of chromosomes multiple times by constantly realigning the new 3' end after each successful telomeric repeat addition. Diseases associated with TERT and hTRC include pulmonary fibrosis, bone marrow failure, and dyskeratinosis congentia (which is also associated with mutations in NOP10, hTRC, and dyskerin).

division. Telomeres are composed of thousands of hexanucleotide repeats (TTAGGG) which end in a G-rich 3' overhang of 30-300 nucleotides long (298,299). These defend against nucleotide loss during DNA replication and prevention of end-to-end fusion during chromosome segregation in late metaphase and anaphase. These repeats are associated with a complex of six proteins called shelterin, which prevents the chromosomal ends from being recognized as DNA double-stranded breaks by DNA repair machinery (300).

Shelterin is composed of TTAGGG binding factors, telomeric repeat-binding factors 1 and 2 (TRF1 and TRF2), protection of telomeres 1 (POT1), repressor/activator protein 1 (RAP1), TRF1-interacting nuclear factor 2 (TIN2) and TIN2 interacting protein 1 (TPP1) (Figure 13 A) (300,301). The double stranded repeats are bound by TRF1 and 2 whereas the single stranded repeats are bound by POT1 which heterodimerizes with TPP1 (301,302). TIN2 serves as an adaptor protein, which creates a link between the TRF1/TRF2 and TPP1/POT1 heterodimers (300-302). Each member is required since deletion studies of each protein decrease the function of sheltrin and increased susceptibility by the DNA damage response (300-302). Telomeres can also form telomeric loops, with the 3' overhang displacing the upstream telomeric region, forming a so-called displaced loop (D-loop) (302,303). Another capping structure is a four-stranded DNA structure called a G-quadraplex which is derived from the folding of single stranded DNA containing tandem repeats of 3-4 consecutive guanines to form stacked tetrads of guanines that are stabilized by Hoogsteen base pairing and cation coordination (303). Both of these structures depend on the activity of the RTEL1 helicase (303). The gradual erosion of telomeres generates genomic instability and this in turn activates ATM/ATR kinases that promote p53 dependent apoptosis and cellular senescence (188,189,303).

When cells become senescent, they become very large, flat, and express high levels of β-galactosidase (302,303). Telomere longevity and renewal is crucial and cancer cells achieve this by primarily activating the enzyme

telomerase. Initially discovered by Elizabeth Blackburn, this enzyme is highly expressed in germ line cells but is repressed in somatic and differentiated cells. Cancer cells upregulate telomerase in order to stabilize telomeric longevity and acquire replicative immortality (304). Telomerase is composed of two molecules each of telomerase reverse transcriptase (hTERT) telomerase RNA component (hTERC), and dyskerin (Figure 13 B) (304). HTERT is a reverse transcriptase, which allows DNA to be transcribed from an RNA template (304). Telomerase carries its own unique RNA template, which is hTERC, and is approximately 451 nucleotides in length (304). TERT uses the template region of TERC (3'CAAUCCCAAUC-5') to add TTAGGG repeats to the 3' end of chromosomes multiple times by constantly realigning the new 3' end after each successful telomeric repeat addition (304). Telomerase is often inactivated in premalignant cancer cells and activated in malignant cells and the rationale behind this is that cancer cells need to acquire mutations that are favorable for malignancy and then activate telomerase to stabilize and perpetuate the mutated malignant phenotype (304,305). Telomerase activation has been observed in about 90% of human tumors and other pathways such as alternative lengthening of telomeres (ALT) exist in tumor and therefore bypassing the need for of telomerase (305). Along with telomere elongation, TERT participates in other cellular activities such as gene expression, DNA repair and apoptosis that can enhance cancer progression (305). Although telomerase overexpression is often associated with a transformed and malignant phenotype, telomerase overexpression itself does not

confer the transformed phenotype as many “normal” immortalized cells also overexpress telomerase. For example, MCF10A mammary epithelial cell line and the pancreatic ductal epithelium cell line HDPE (human ductal pancreatic epithelium) have been immortalized by telomerase and are used as models for “normal” cells (306,307). In some instances, impaired TERT function can enhance carcinogenesis in a certain biological context, such as when coupled with defective p53 (188,189,305). Although TERT does not confer the transformed phenotype and its role in cancer is somewhat paradoxical, it is more often than not a critical component in cancer cell progression and it expression can enhance malignancy by conferring replicative immortality.

Inducing angiogenesis

Vascular endothelial growth factor (VEGF)

Rapidly growing tumors have a very high demand for nutrients, oxygen, and other biological resources to promote an anabolic environment and avoid becoming excessively stressed to induced necrotic cell death (308). Cancer cells in the center of a tumor mass will become hypoxic and necrotic and another crucial hallmark of cancer is the acquisition by a cancer cell to have sustained blood flow that delivers nutrients and oxygen, and removes wastes and CO_2 (308). This is accomplished by inducing angiogenesis, which is the formation of new blood vessels from the pre-existing vasculature and this process can also be transiently activated during wound healing (308). However, sustained and perpetual

induction of angiogenesis is required for tumors and suppression of angiogenesis leads to anoxic tumor necrosis as well as tumor dormancy, which is evidenced by asymptomatic microscopic tumors that are commonly observed in clinical settings. In 1971, Folkman first described the interaction between vascular endothelium and tumors and postulated the requirement of angiogenic growth factors (309). The most fundamental gene involved in angiogenesis is vascular endothelial growth factor (VEGF), which is also important in normal physiology and embryological development which also need the formation of new blood vessels (Figure 14) (310). VEGF and angiogenesis are also required after vessel and ischemic injury during exercise and to bypass blocked vessels to prevent ischemic injury (310). VEGF is a member of the platelet-derived growth factor (PDGF) family of cysteine-knot growth factors and was first identified in guinea pigs by Dr. Sanger in 1983 and originally labeled the “vascular permeability factor” (311). VEGF has five isoforms: VEGFA, B, C, D, and Placental growth factor (PIGF) (310). VEGF-A is the most physiologically relevant isoform of VEGF in cancer and it induces angiogenesis, migration, and proliferation of endothelial cells; VEGF also upregulates MMPs, and increases expression of the vitronectin receptor (integrin αvβ3, the known receptor for vitronectin, a critical component of the ECM important for angiogenesis and metastasis) (312,313). VEGF also indirectly induces vasodilation because of nitric oxide (NO) production by stimulated eNOS (endothelial nitric oxide synthase) activity, which attracts macrophages, neutrophils and other granulocytes, and produces growth factors

that can be exploited by the tumor (314,315). VEGF-C VEGF-D and PIGF also contribute to angiogenesis but to a lesser degree. PIGF and VEGF-B are more involved in embryological development, VEGF-C in lymphangiogenesis, and VEGF-D in the vascularization of the lung bronchioles. VEGF protein activity requires initial binding to their cognate receptors, namely: VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4) (315,316). These receptors are RTKs and function in a very similar fashion; they autophosphorylate when activated by ligand and can stimulate downstream PI3K/Akt and Ras/Raf/MEK/Erk pathways. Of the three receptors, VEGFR2 seems to mediate all the downstream responses required for angiogenesis, with VEGFR1 being able to modulate VEGFR2 signaling both positively and negatively (315,316). VEGFR1 can in some contexts serve as a decoy and sequester VEGF, preventing it from binding to VEGFR2. VEGFR3 mediates lymphangiogenesis and its cognate ligand is VEGF-C (315,316).

VEGF expression can be triggered by hypoxia, inflammation, and other factors associated with the tumor microenvironment (315-317). Transcription factors such as NFκB and Sp1 activate transcription of VEGF in breast lung and pancreatic tumor cells and it was demonstrated that hormone activation of VEGF transcription was due to estrogen receptor α (ERα) interacting with Sp1 bound to GC rich regions within the VEGF promoter (317). Tumors *in vivo* secrete VEGF ligands which bind to VEGFRs and initiate mitogenesis within the stromal cells by induction of PI3K/AKT, Ras, and PKC signaling, actin remodeling through MKK

3/6 signaling and Hsp27, and activation of FAK through the vitronectin receptor, and vascular permeability by production of IP3 (318). IP3

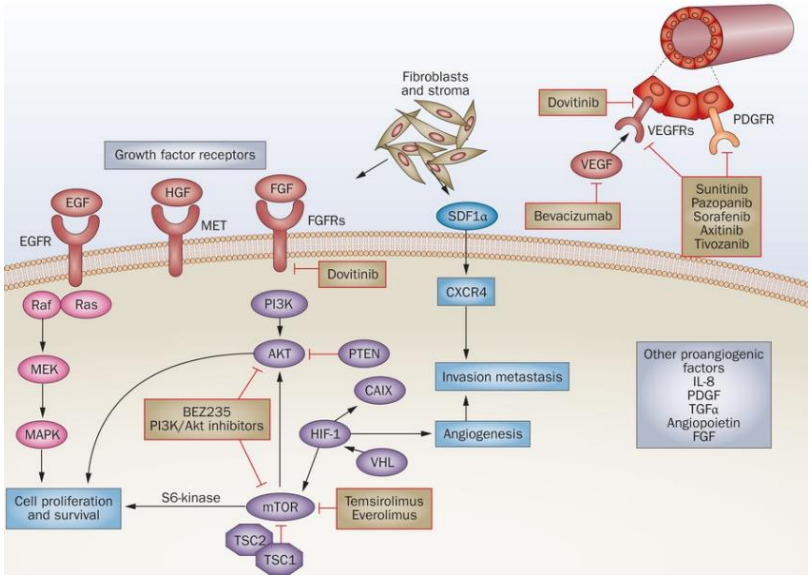

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Figure 14: Angiogenesis. Angiogenesis is a critical part of cancer progression as well as normal physiological function. In cancer angiogenesis is induced to form new blood vessels to hypoxic tumors by VEGF (vascular endothelial growth factor) and its cognate receptors (VEGFR1, VEGFR2, and VEGFR3). Angiogenesis is also mediated by platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) and their respective receptors PDGFR and FGFR respectively. Other angiogeneic factors include IL-8, TGFα, and angiopoietin.

stimulates IP3 and ryanodine receptors on the surface of the endoplasmic reticulum (ER) which release calcium and stimulate eNOS and prostaglandins, resulting in increased vascular permeability (319). However, release of endogenous angiogenesis inhibitors such as angiostatin, endostatin and thrombospondin-1 (TSP-1) and also transcriptional repressors of VEGF such as ZNF24, can counterbalance the effects of VEGF on endothelial cells. VEGF can

also be sequestered by proteoglycans within the ECM and are only released by the action of MMPs (320,321). In 1990, Folkman and Hanahan proposed the "angiogenic switch" concept that described an equilibrium between an angiogenic inducing and an angiogenic-repressing environment, that explain how tumors proceed from a state of dormancy to malignancy and activation of angiogenesis is a critical component of the process (322). The angiogenic switch can occur at any stage of tumorigenesis and development and it is dependent on the type of tumor and the communication within the tumor's microenvironment, which includes many types of myeloid and inflammatory cells synergistically promoting angiogenesis with tumor cells (322). Furthermore, certain bone marrow-derived cells can differentiate into endothelial cells and pericytes to form tumor vasculature. Monoclonal antibodies such as bevacizumab and ranibizumab and drugs like afibercept are agents that target VEGF and show promise for treating retinoblastoma, breast cancer, and RCC (323-325).

HIF-1 and VHL

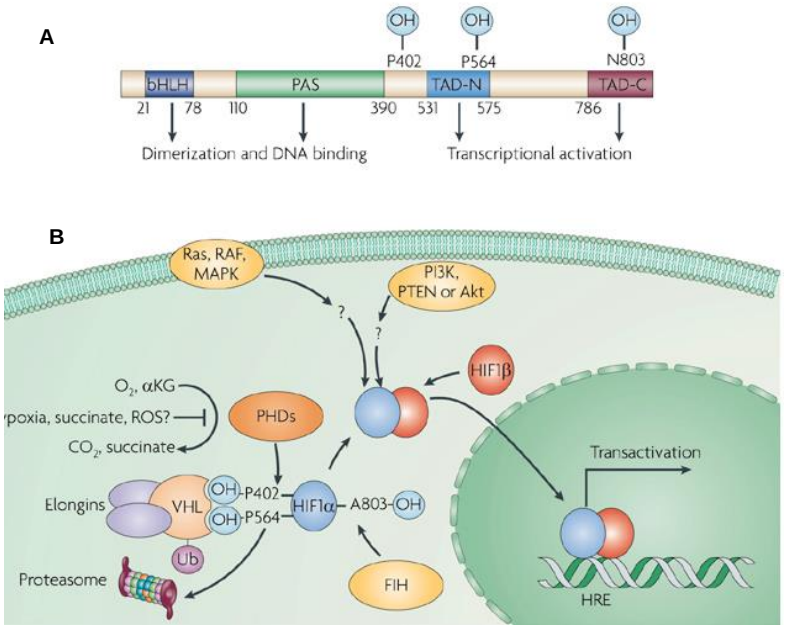

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Figure 15: HIF-1. A) The structure of HIF-1 (HIF-1α and HIF-1β), which are members of the PAS (period, ARNT, and singleminded) family of transcription factors. The basic helix-loop-helix (bHLH) and PAS domain confer DNA binding and dimerization. The C-terminal region contains transactivational domains (TAD-N and TAD-C) which are necessary for transcriptional activation and contain sites for hydroxylation by prolyl hydroxylase domain-containing enxymes (PHDs), which included P402 and P564. B) These hydroxylations occur in normoxic, unstressed conditions and enable binding by the E3 ubiquitin ligase von-hippel lindau (VHL), which promotes proteasome-dependent degradation. In hypoxic conditions, PHDs are not activated and HIF-1α translocates to the nucleus to induce hypoxia inducible genes.

As stated above, hypoxia is a potent driver of angiogenesis and hypoxia-induced angiogenesis is orchestrated by the protein known as HIF (hypoxia inducible factor)-1α. It is a subunit of a heterodimeric complex, the other member being HIF-1β (also known as ARNT or aryl hydrocarbon receptor nuclear translocator) (326). HIF-1β is a PAS basic helix-loop-helix (bHLH) containing

protein (Figure 15A) and is the master regulator of the hypoxia inducible response (327). Dysregulation of HIF-1α has serious consequences in a cancer context. One of the most important regulators of HIF-1α is the E3 ubiquitin ligase VHL (Von Hippel-Lindau) tumor suppressor, which is often mutated in cancers, especially RCC, and is the cause of the eponymous disease von Hippel-Lindau disease (328). This disease is characterized by the spontaneous generation of hemangioblastomas, angiomatosis, pheochromocytomas, pancreatic and glomerulic cysts, and RCC (renal cell carcinoma (328-330). The VHL gene is found on the short arm of chromosome 3 and over 1500 germline and somatic mutations are associated with the disease, which is autosomal dominant (327). Many of the mutations (30-40%) in the VHL gene consist of 50-250 kb deletion mutations that produce a truncated protein, the remaining of mutations cause truncated proteins by nonsense, insertional/deletional, and splice site mutations (328-330). Cells that express the normal copy in heterozygous individuals are healthy, however cells that express the mutated version have epistatic penetrance that results in formation of benign neoplasms within the kidney and pancreas (329,330). In normal physiological conditions HIF-1α is hydroxylated by prolyl hydroxylase domain containing enzymes (PHDs) and this enables association and recognition of HIF-1α by the VHL protein (Figure 15B) (326-330). VHL is a component of an E3 ligase complex that includes ECV (elongins, Cul2, VHL) and RBX and this association results in proteasome-dependent degradation of HIF-1α (326-330). However, when VHL is mutated and cannot recognize HIF-1α even

under normoxic conditions, this results in hyper-angiogenesis and increased carcinogenesis (326-330).

HIF-1 and MITF

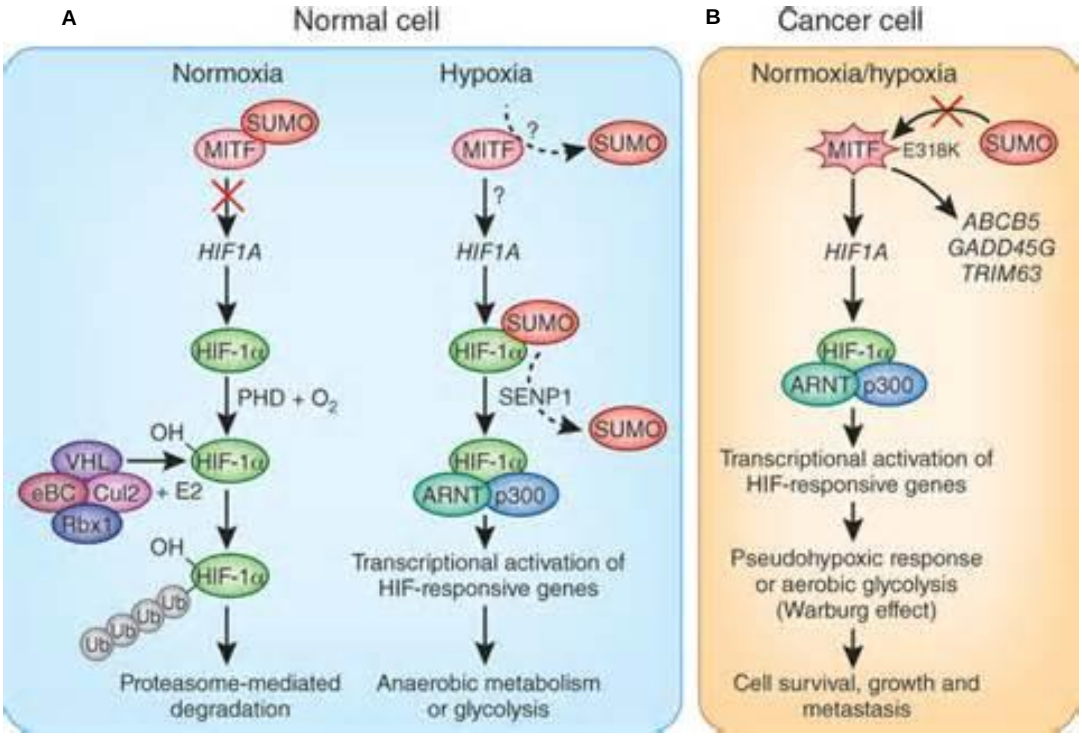

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Figure 16: The role of MITF on HIF-1α stability. A) Under hypoxic conditions, MITF promotes association of HIF-1α with ARNT and the histone acetyl transferase (HAT) enzyme p300 after the reversible de-sumoylation of HIF-1α by SENP1 (Sentrin specific protease 1). B) Mutations of MITF also enhances carcinogenesis MITF also induces expression of the drug pumping protein ABCB5 (an ATP binding cassette protein drug that is implicated in chemotherapeutic resistance in melanoma and RCC), TRIM63 (an E3 ligase that is implicated in increased malignancy in RCC and breast cancer) and GADD45G (Growth Arrest and DNA Damage γ), which is important in DNA damage repair.

Another protein called MITF (microphthalmia associated transcription factor) is bound by a SUMO (small ubiquitin like modifier) molecule, which has low affinity for HIF-1α (331). Under hypoxic conditions, MITF promotes association of

HIF-1α with ARNT and the histone acetyl transferase (HAT) enzyme p300 after the reversible de-sumoylation of HIF-1α by SENP1 (Sentrin specific protease 1) (Figure 16A) (331). Mutations of MITF also enhance carcinogenesis, for example a mutation of MITF that frequently occurs in RCC and melanoma is E318K, and this inhibits conjugation of SUMO to MITF, resulting in activation of HIF-1α and induction of angiogenesis, aerobic glycolysis, cell growth and metastasis (Figure 16B) (331). MITF also induces expression of the drug pumping protein ABCB5 (an ATP binding cassette protein drug that is implicated in chemotherapeutic resistance in melanoma and RCC), TRIM63, an E3 ligase that is implicated in increased malignancy in RCC and breast cancer and GADD45G (Growth Arrest and DNA Damage γ), which is important in DNA damage repair (331).

VEGF and NFκB are potent transcriptional activators of HIF-1α along with Stat3. HIF-1α is also heavily regulated at the post transcriptional and posttranslational level. RNA binding proteins such as PTB (polypyrimidine tract binding protein 1) and HuR (human antigen R) bind and stabilize HIF-1α mRNA (332,333). HIF-1α is also regulated by miRNAs (micro RNAs) which are a class of non-coding small (~22nt long) RNAs that regulate mRNA stability by either inhibiting translation or promoting degradation by binding to the 3'UTR of a targeted mRNA (334). MiRNAs can repress or stabilize HIF-1α transcript by either directly binding to its transcript or by repressing expression of HIF-1α repressors (334). The miRNA machinery reported to repress HIF-1α mRNA include miR-519c, miR-107, miR-155, miR-20b, and miR-199, miR-17-92 (335-340). Some

miRs including miR-424 and miR-210 target negative regulators of HIF-1, Cul2 and GPD1L respectively (341,342). HIF-1α also has IRES (internal ribosomal entry sites) and is sensitive to calcium signaling (by activation of calcineurin and RACK1) which increase stability of HIF-1α (343). Along with PHDs and VHL, HIF-1α is degraded by proteins OS-9 (osteosarcoma amplified 9) which promotes its proteasome-dependent degradation, and the protein SSAT2 (spermidine/spermine N1-acetyltransferase 2), which stabilizes the interaction of HIF-1α and the E3 ubiquitin ligase complex (344,345). SSAT can do this both in an oxygen dependent and independent manner (344). Ubiquitination and sumoylation also plays a role in the stability of HIF-1α; sumoylation (catalyzed by RSUME), and de-sumoylation (SENP1, and the enzyme VDU2 de-ubiquitinating HIF-1α) all have stabilizing effects on HIF-1α during hypoxia and the proteins GSK3β and the transcription factor FOXO4 also promote degradation of HIF-1α (346-348).

HIF-1 regulation

During hypoxia in a cancer cell, HIF-1α regulates expression of VEGF and also enthryopoietin, which are both powerful drivers of not only angiogenesis but also erythropoiesis, and thus increases blood delivery to cancer cells (326,327). HIF-1α also upregulates the transcription of genes involved in cell proliferation and migration, as well as those involved in glucose and iron metabolism and these include hexokinase2, glucose transporter GLUT1, monocarboxylate transporter 4 (MCT4), and REDD1 (a negative feedback loop protein that inhibits mTOR by

stimulating TSC1/2) and BNIP3 (326,327,334-340). Thus, VEGF and HIF-1α represent attractive cancer therapeutic targets for inhibiting tumor angiogenesis at the primary site and also for cancer cell metastasis at distal sites.

Activating invasion and metastasis

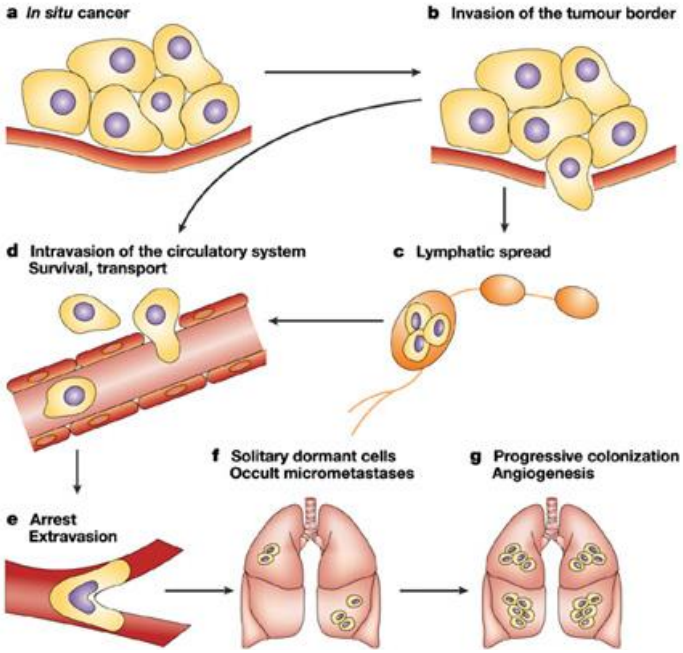

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Figure 17: Steps of metastasis. Cancer cells that have sustained unregulated cell proliferation grow within an encapsulated barrier formin a carcinoma *in situ.* As a tumor acquires a malignant phenotype it undergoes EMT and overexpresses proteases such as MMPs, which degrade the basement membrane. These cells then invade the ECM and intravasate into blood vessels and spread to lymphatic tissue and distal organs. These cells then undergo MET and form metastatic lesions.

The most definitive feature of a malignant tumor is the ability to separate from the primary tumor, migrate, invade the surrounding tissue, and metastasize

to distal sites. Cells can be anaplastic or dysplastic or have all the above capabilities, but still be a benign tumor or a ductal carcinoma in situ (DCIS) and never become malignant until they acquire the ability to migrate invade and metastasize (Figure 17) (349). Metastasis is the cause of 90% of cancer mortality and acquisition of this characteristic can occur early and it is estimated that 60-70% of cancer patients have overt metastases at diagnosis (51,349,350). Malignant cancer cells undergo EMT and begin to invade by invading the surrounding tissues, degrading extracellular matrix by upregulation expression of MMPs, invade lymph nodes, and transcoelomically spread (55,349,350). Tumor cells will invade the lymphatic networks and intravastate blood vessels in order to disseminate to distal tissues and colonize, and form metastatic lesions. Angiogenesis enables cancer cells to disseminate in blood circulation and some tumor cells accomplish this by secreting VEGF-C and VEGF-D, which bind to VEGFR3, induce lymphangiogenesis, and enhance metastasis (315,316,351). Metastasis to the lymph nodes is physiologically distinct than the type observed by homeostatic chemokines CXCL12-CXCR4), which challenges the common perception that cancer cells must first metastasize to the lymph nodes then to distant organs, as the homeostatic axis relies on the “seed and soil” hypothesis mentioned earlier (352,353). Invasion and metastasis use many of the same gene expression cassettes to increase cell motility and cell migration.

Cadherins, β-catenin, and EMT

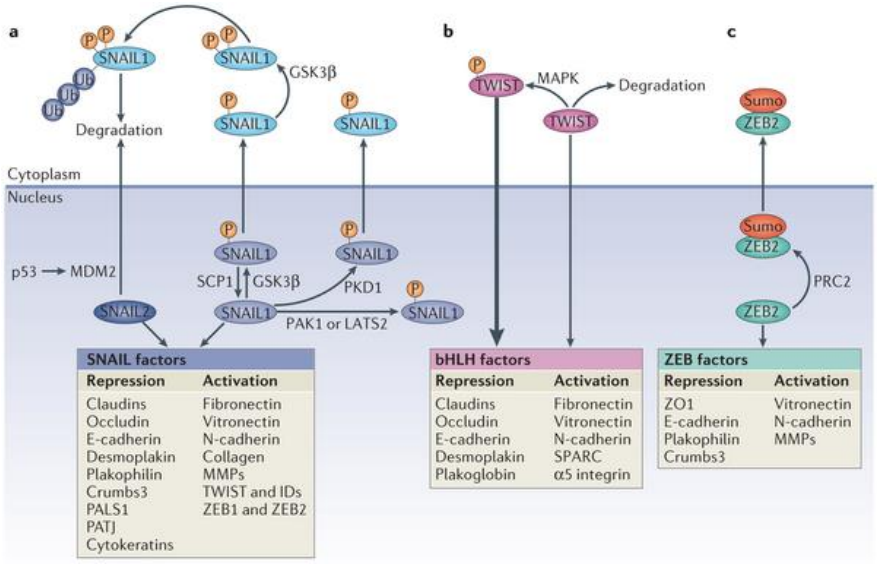

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Figure 18: Steps of EMT. EMT is a critical process cancer cells must undergo in order to become metastatic and colonize distal organs. This involves not only changes in morphology (fibroblast morphology, loss of apical to basal polarity, formation of invasipodia, and loss of cell to cell adhesion) but also decreased expression of epithelial markers (such as E-cadherin, occluding, and ZO-1) and upregulation of mesenchymal markers (N-cadherin, vimentin, T-cadhein, collagen 1. The transcription factor β-catenin also becomes unbound from the membrane with E-cadherins and translocates to the nucleus to transcribe EMT associated genes such as slug and snail.

The paramount phenotypic change in metastatic cancer cell is the loss of polarity and the loss of cellular and basement membrane attachment and this involves E-cadherin (Figure 18) (354). E-cadherin is a calcium dependent adhesion molecule that forms vast networks that span the cell membrane and interact with extracellular domains of other e-cadherin molecules from adjacent cells. Intracellularly, the E-cadherin networks are stabilized by vinculins and α-

actinin, and bind to p120-catenin (δ-catenin), plakoglobin (γ-catenin), β-catenin, and α-catenin (indirectly through plakoglobin and β-catenin) (354,355). E-cadherin is a natural repressor of migration and invasion and cancer cells must inhibit its expression in order to become invasive (354). This is accomplished by upregulating expression of EMT transcription factors, such as Snail, Slug, ZEB, and Twist family members, which directly repress expression of E-cadherin and induce expression of mesenchymal proteins N-cadherin and vimentin (354,355). Cells also lose expression of cytokeratin and Zonula occludins (ZO) molecules and upregulate expression smooth muscle actin α (ACTA2), fibroblast specific protein (FSP1), Type 1 collagen, and fibronectin (354-356). Mutations of E-cadherin are quite rare in many cancers but it is present in 50% of all breast cancers. The cytosolic location of β-catenin is also changed in a metastatic cell as it translocates to the nucleus and upregulates expression of pro-survival genes (including c-Myc, cyclinD1, ASCL2, ITF-2B, and pluripotency genes oct4, sox2, and nanog).

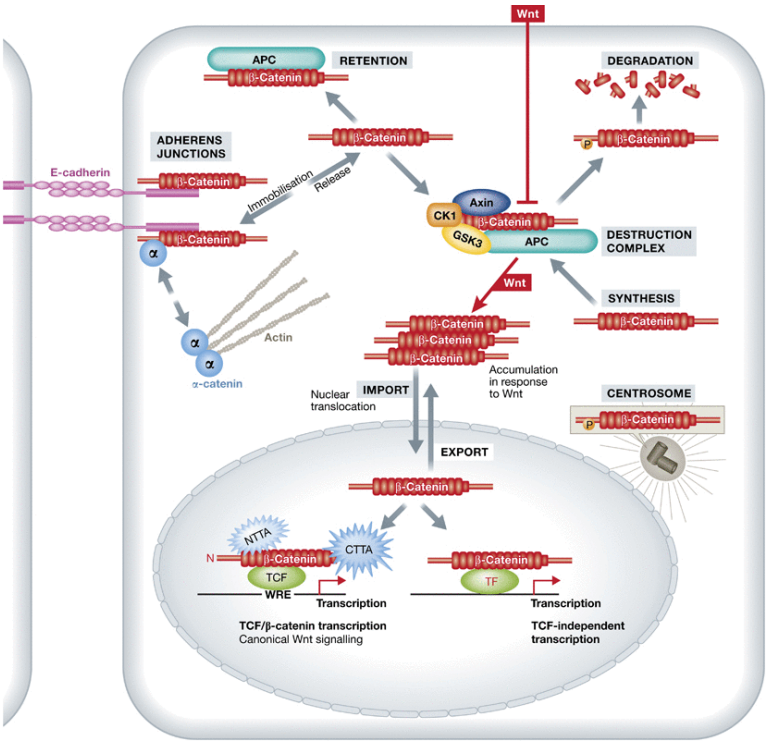

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Figure 19: β-catenin subcellular localization and it stability. The Wnt signaling pathway is a major mediator of β-catenin transcriptional activity and its stability. In resting conditions, β-catenin is complexed with APC, Axin, and kinases GSK3-β (glycogen synthase kinase 3β) and CK1α (casein kinase 1), with the latter two phosphorylating β-catenin at S33 and S37 respectively. This enables β-catenin to be recognized by βTrCP, and ubiquitinated for proteasome-depednet degradation. In epithelial cells β-catenin is also associated at adherens junctions with E-cadherin. Upon stimulation by Wnt, β-catenin dissociates from the complex and translocates to the nucleus to serve as a transcription factor for TCF-dependent genes.

Dissociation of β-catenin from the adherens junction network promotes cell motility and loss of cellular attachments (357). Free cytosolic β-catenin is also tightly regulated in the cytosol and it is a potent activator of Wnt signaling and this is particularily important in colorectal and prostate cancers (357). In the absence of ligand, APC (adenomatosis polyposis coli), Axin, GSK3β, and CK1α sequester

β-catenin in the cytosol, where it is hyperphosphorylated and subsequently ubiquitinated by β-TrCP (which contains BRTC and SKP1-CUL1-F-box) and then degraded by the proteasome (Figure 19) (357,358). However, when Wnt binds to Wnt receptor LRP-5/6 and frizzled, the phosphoprotein disheveled (dsh) is activated and it disrupts the destruction complex as CK1α (casein kinase 1α) phosphorylates the cytoplasmic tail of LRP-5/6 which promotes Axin binding (357,358). This permits β-catenin to accumulate in the nucleus and subsequently induce a cellular response (EMT like responses and invasion) by interacting with other transcription factors TCF/LEF (T-cell factor/lymphoid enhancing factor) (359). Mutations in β-catenin are common in prostate and colorectal cancers but rarer in other kinds of cancers.

EMT transcription factors such as ZEB, snail, and slug are also important for wound healing and embryological morphogenesis (354,355). There are three distinct forms of EMT, the first two being embryological development, and wound healing respectively, with the third type being related to malignant cancer (354,355). EMT can be transiently or stably activated in both epithelial and non-epithelial tumor types, promoting an invasive and metastatic phenotype in these tumors (354,355). This is accompanied by a loss of cell polarity (which is accompanied by loss to attachment of the basement membrane by hemidesmosomes), loss of zonula and adherens junction and desmosomes, increased motility, increased expression of matrix degrading enzymes such as MMPs, and acquisition of a fibroblastic or amoeboid morphology (354,355).

Blood vessel intravasation and mesenchymal to epithelial transition

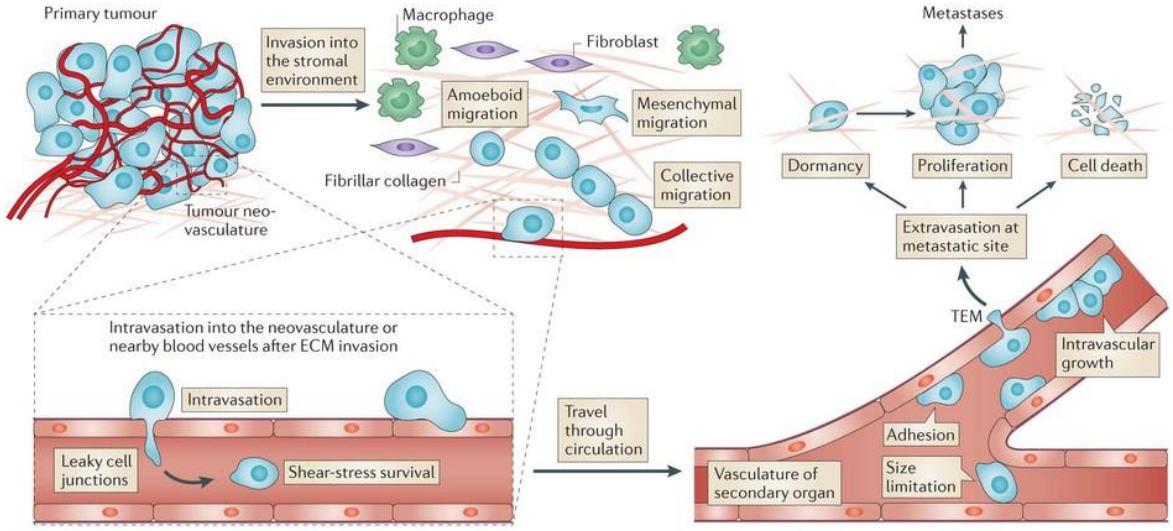

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Figure 20: Blood vessel intravasation and MET. Model highlighting the key morphological and biological features of cancer cell invasion and metastasis. A benign tumor progressesto a malignant state and then becomes invasive. This is accompanied by expression of matrix metalloproteinases, ADAMs, and undergoin EMT. The cells then migrate and intravaste blood vessels to become part of systemic circulation. The cancer cells then extravasate the blood vessels into a distal tissue and then undergo MET (mesenchymal to epithelial transition) and form micrometastases and colonize distal tissues.

Tumor cells also stimulate stromal cells such as mesenchymal stem cells (MSCs) and tumor associated macrophages, which foster the malignant behavior in these cancer cells by being a garrison for growth promoting factors and chemokines such as CCL5, IL-8, and MMPs (Figure 20) (360). Another feature of EMT in tumors is that this process is reversible and this is critical for establishing metastatic sites (360). Cancer cells must revert to their epithelial type by undergoing mesenchymal to epithelial transition (MET) (354,355,360). The

process of metastasis is very inefficient and cancer cells must adapt to harsh conditions and selective pressures in order to become an established metastatic lesion (361). Indeed, it is estimated that millions of tumor cells enter the circulation daily and less than 0.01% of these circulating tumor cells successfully extravasate a distal tissue and initiate metastasis (361). An ostensible impediment is that most cancer cells from a primary tumor poorly adapt to a foreign environment without the support of the stroma. This is apparent by the prevalence of dormant micrometastases that never overcome the initial hurdle to become macroscopic secondary tumors (51,354,361). Some primary tumors also release angiogenic inhibitors such as angiostatin and endostatin that suppress growth of metastatic lesions, which underscores the competitive and selective nature of metastasis and only a small fraction from a heterogeneous neoplastic population are selected to form secondary tumors (51,354,361). However, depending on the origin of the primary tumor cells the secondary tissue site can be hospitable to the newly colonizing cells. As stated earlier, colorectal cells preferentially metastasize to liver and lung. In breast cancer, metastasis is observed in bones and lung, in malignant melanoma the brain, and in stomach cancer the ovaries (i.e. krukenberg tumor) (51-53,360,361). This non-random organotropism of cancer metastasis is explained by Paget's "seed and soil" theory as mentioned above (362). This theory was not met uncontested since Ewing proposed that mechanical forces like blood flow and the diameter of blood vessels, and blood flow between the primary tumor and putative metastatic sites dictated the specificity of the metastatic site

(362,363). For example, the lung is a favorable milieu for a metastasis due to its dense vascular surface area, whereas the liver attracts colorectal tumor cells due to the mesenteric circulation pattern. Ewing's hypothesis was dominant for over 50 years until it was disproven by molecular evidence within the past 30 years (362,363). Metastasis is regulated by communication between the seeding tumor cells and the tumor microenvironment that will sow and harvest that tumor cell into a metastasis (362,363). For example, breast cancer cells express chemokine receptor CXCR4 and metastasize to bone marrow, which express its cognate ligand CXCL12 (364). Colorectal cancer, exhibits high expression of TGF-α and metastases to the liver which express the EGFR (365). Moreover, some of these molecular determinants are chemokines, which navigate cancer cell chemotaxis like the CXCL12-CXCR4 axis (364). These chemokines are constitutively expressed in specific tissues and therefore called homeostatic chemokines. The process of metastasis is very selective, complex and depends on an orchestration of multiple molecular components from the tumor microenvironment, the primary tumor, and the parenchyma itself and the molecular interplays of metastasis are still not consummately understood and are still being rigorously investigated.

The extracellular matrix (ECM) and integrins

Not all tumor cells undergo these EMT changes simultaneously and it is usually only observed in cells residing at the invasive front of the tumor that are adjacent to the ECM (51,52,366). This suggests that the ECM plays a pivotal role in triggering these physiological and morphological changes associated with

invasiveness and motility. The extracellular matrix (ECM) and the intracellular milieu are mutually dependent on one another and can send signals to change the shape, arrangement, chemical makeup, and the dynamics of the environment (Figure 21) (51,52). The extracellular matrix includes the interstitial matrix and the basement membrane, which is composed of type IV collagen, entactin, nidogen, and fibronectin, and vitronectin (51,52). The interstitial matrix is rich in fibrillary

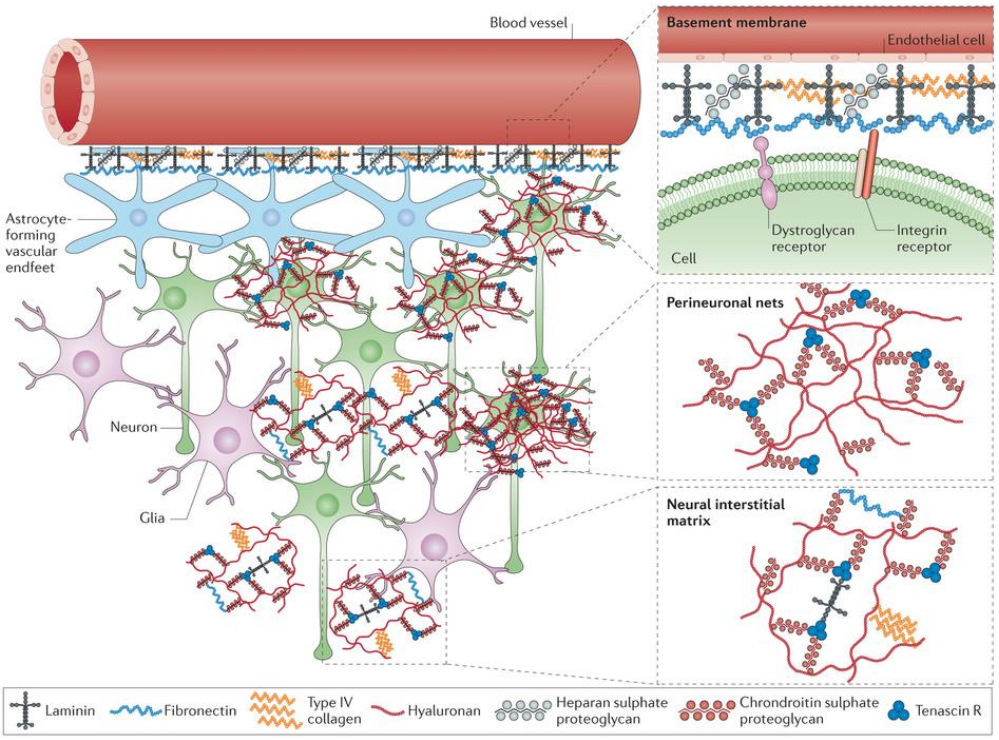

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Figure 21: The extracellular matrix. The complex and integrative network of proteins called the extracellular matrix (ECM). The ECM interacts with surface membrane proteins which include integrins, syndecans, glypicans, and collagen. These surface membrane proteins serve as conduits which mediate "inside-out" and "outside-in" communication, meaning the intracellular and extracellular environment respond to simuli from the opposite side. THE ECM is a diverse network of glycoproteins, proteoglycans, hyaluronic acid, perlecans, vitronectin, fibronectin, FACIT (Fibril Associated Collagens with Interrupted Triple helices) collagens (which include collagen type, IX, XII, XIV, XIX, and XXI, fibrallar collagen.

collagens (Type I, II, III, V, XI), tenasin C, highly charged, hydrated fibronectin (heparin sulfate and syndecans) Normal epithelial cells are attached to the basement membrane through hemidesmosomes and the ECM provides growth factors and growth signals from the outside (51,52,366). The ECM is very dynamic and can have varying degrees of elasticity and stiffness, which correlate with the tumorigenic propensity of a transformed cell. One of the factors that contributes to ECM stiffening is the enzyme lysyl oxidase (LOX) (367). This enzyme induces crosslink within collagen fibers, which leads to increased ECM stiffening. When the ECM stiffens, this mechanosignaling is transduced to the cell, which becomes more rigid and forms cortical actin tension fibers (366). Proteins known as integrins sense these mechanical signals and integrins form heterodimers on the cell surface and can communicate with the ECM by binding to specific components of the ECM. Specifically, α5β1 integrin heterodimers bind to fibronectin at the FnIII $_{9\text{-}10}$ which contain the RGD motif recognized by β1 integrin (366,368). β1-Integrin and its role in tumorigenesis, migration, and malignancy is ostensible and well documented (368,369). β1-Integrin has multiple binding partners of the α-integrin family, which include 18 members and the β family contains eight isoforms. β and α chains form heterodimers with 24 different heterodimers that can form and promote focal adhesion complexes (FACs) (369). Along with fibronectin, the proteins type 1 collagen (binds to α2β1 integrin) vitronectin (binds αvβ3) and laminin (α6β4) are physiologically relevant, in

pancreatic, lung, colorectal, breast cancers, as well as temozolomide refractory glioblastoma multiforme (GBM) (type 1 collagen receptor and fibronectin receptor) (370-373). Different heterodimer combinations recognize different components of the ECM. α1β5 heterodimers recognize a specific region within fibronectin domain Fn III that contains the RGD (arginine, glycine, aspartate motif), whereas the αVβ3 heterodimer recognizes vitronectin and vitronectin recognizes αVβ3 via its somatomedin domain (368,369,370-373). Association of these heterodimers with the ECM promotes the formation of focal adhesions. β1-Integrin is an integral component of conveys signals between the ECM and the intracellular milieu (369). Specifically, β1-integrin can recruit focal adhesion kinase (FAK) which can autophosphorylate and cause recruitment of Src and Src kinase family members as well as p130cas (which is a substrate for Src (369-373). Src can phosphorylate FAK and further recruit other protein and actin-associated proteins such as vinculin and talin. Upon β1-Integrin heterodimerization and binding to the ECM (i.e. fibronectin), there is association of Integrin binding proteins such as paxillin, which enables recruitment of the focal adhesion kinase (FAK) (369-373).

Focal adhesion complexes and cytoskeletal activation

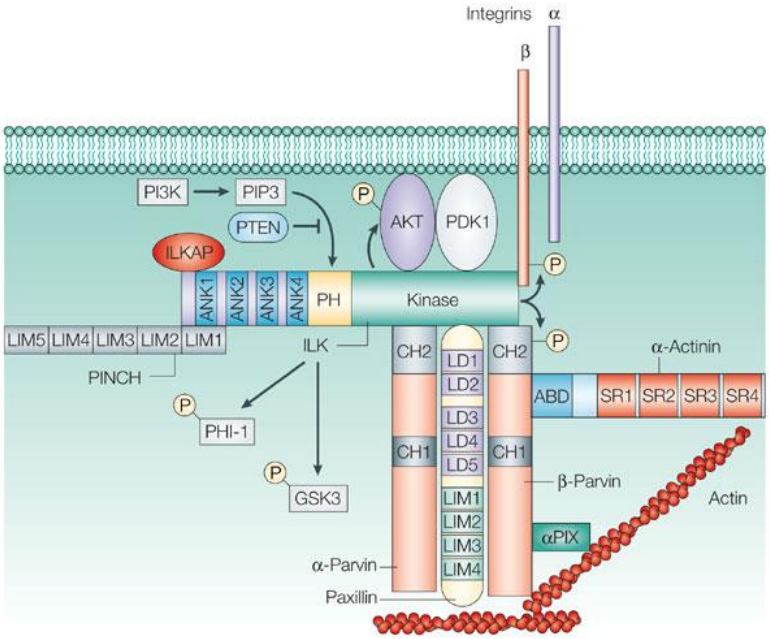

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Figure 22: Focal adhesion complexes (FAC). Integrins are in the inactive state until stimulation by binding to extracellular components (such as fibronectin and vitronectin). Upon ligand activation Paxillon binds to integrin cytoplasmic tails and recruits FAK (focal adhesion kinase). FAK autophosphorylates at Y397 and this promotes binding of Src kinases (such as src, fyn, yes). Talin and vinculin also bind to paxillin and promotes association of β-actin and formation of stress fibers. Integrin-linked kinase (ILK) can also bind to the cytoplasmic tails of integrin and promote Parvin and PINCH. These also activate PI3K/Akt and Ras pathways and promote cell proliferation and actin stress fiber promotion through PKC activated Rac, RhoA, and cdc42.

Upon association of FAK, FAK becomes autophosphorylated at tyrosine residue 397. FAK contains two domains: A N-terminal FERM domain (4.1 protein, ezrin, radixin, moesin domain, which enables association with actin and actin binding proteins as well as actin like proteins) which serves as an auto inhibitory domain and a C-terminal kinase and FAT (focal adhesion targeting) domain

(Figure 22) (369-373). These two regions are joined together by a flexible linker region that enables the FERM domain to inhibit the kinase domain of FAK by binding to it (369-373). Autophosphorylation of FAK enables Src kinase to associate with FAK through its SH2 domain and phosphorylate FAK on other sites (369-373). This opens up other docking sites for proteins that contain SH2 (which recognizes phosphotyrosine residues) and SH3 (which recognizes proline rich regions) domains within FAK, including Src substrate p130cas (which promotes migration and contains an SH3 domain) ZAP70, SOC3 (369-373). FAK interacts with Talin and vinculin through its FERM domain and the resulting complex associates with actin to promote formation of actin stress fibers (369-373).

TGF-β signaling and metastasis

Tumor cell malignancy and invasion also go hand in hand with a physiological phenomenon known as epithelial to mesenchymal transition EMT (374). A powerful driver of EMT is TGF- β (transforming growth factor β). This includes induction of mesenchymal transcription factors (ZEB, Snail, Slug, Twist) that upregulate changes in cell-to cell contacts through differential expression of cadherins (i.e. decreased E-cadherin and increased N-cadherin (374,375). In early stage cancer, TGF- β is a tumor suppressor and inhibits cancer cell growth and promotion through canonical TGF- β signaling pathways. TGFβRII binds to TGFβRI, phosphorylates the GS loop, which activates its kinase activity (375,376). Through association with SARA (SMAD anchor for receptor activation) phosphorylates Smads 2, 3, which associate with Smad 4, translocate into the

nucleus and induce transcription of tumor suppressor genes. However, in later stage cancer, TGF- β signaling becomes aberrant, and can signal through non-canonical TGF- β pathways. TGF- β has also been shown to induce migration in late stage breast cancer cells (375,376). It has been demonstrated that TGF- β can induce formation of a destructive complex that promote ubiquitination and proteasome-dependent degradation of inhibitory Smad 7. Smad 7 inhibits TGF-β signaling by promotion the endocytic recycling of TGF- β.

All isoforms of TGF- β are dimers that are excreted and are associated with a protein called latency-associated peptide (LAP), which constitutes the small latent complex (SLC) (374,377,378). This complex remains in the cell until it is bound by another protein called Latent TGF-β-Binding Protein (LTBP), forming a larger complex called Large Latent Complex (LLC). LLC is secreted to the ECM (377,378). The LAP contains an RGD motif that is recognized by α5β1 heterodimers that are a part of the focal adhesion complex that was described above (378,379). Upon cell stiffening and actin stress fiber formation, the integrin complex pulls on the LAP, and through this mechanic opening of the LAP TGF-β is released (380). There is an ostensible interplay and connection between ECM/cell stiffening and aberrant TGF- β signaling and their roles in promoting tumor malignancy, which emphasizes the therapeutic potential of drugs that target TGF- β (380,381). The role of TGFβ in the research described in this thesis will be described later. β1-Integrin can also directly stimulate TGF- β signaling through the LAP as described above. β1-Integrin is a very promising drug target but it is

very hard to target due to compensatory mechanisms (i.e. β3-Integrin expression and so-called integrin switching in breast cancer) as well as other compensatory mechanisms (382).

Genomic instability

Epigenetic regulation

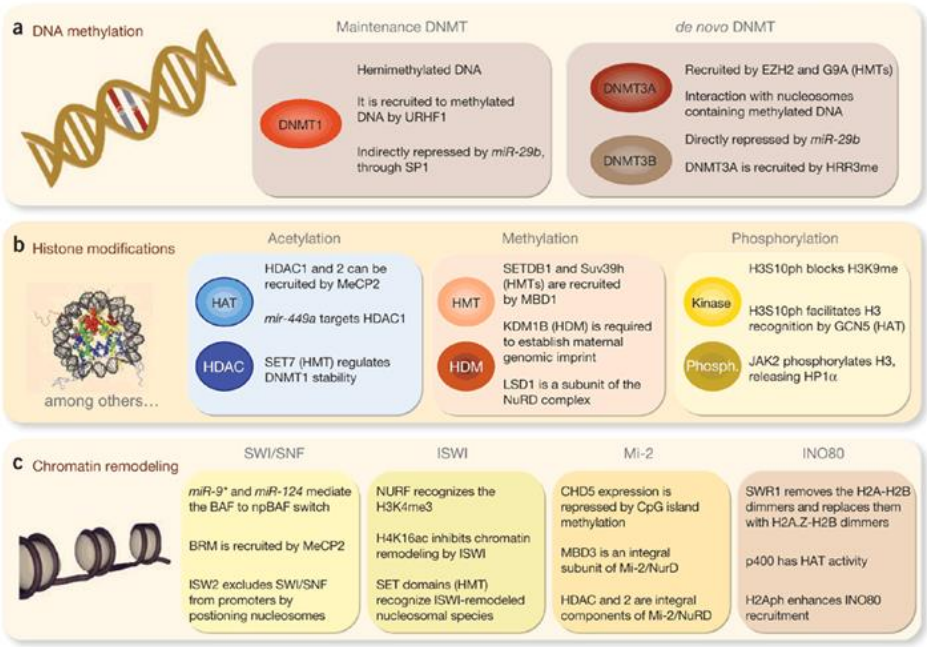

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Figure 23: Epigenetic regulation changes that occur in cancer cells. This involves epigenetic upregulation of oncogenes and epigenetic silencing of tumor suppressor genes (such as CTBP1, p53, SMAD3, and TGFβRII). This is accomplished by direct DNA methylation, nucleosome postioning, histone acetylation (methylation, acetylation, and phosphorylation) on the same gene (*in cis*) or different genetic loci *(in trans).*

Genomic instability refers to loss of DNA integrity, chromosomal aberrations (deletions, translocations, and insertions), epigenetic and genetic

aberrations, high mutations rates, unsustainable single nucleotide polymorphisms, and any other accumulative damage that impairs genomic integrity (383-385). These can include mutation in the primary nucleic acid sequence itself, in the machinery that regulates its expression (DNA methyl transferases (DNMT), histone acetyl transferases (HATs), histone deacetylases (HDAC)) (Figure 24), chromosomal instability (i.e. Philadelphia chromosome observed in CML), microsatellite instability, transposon instability, and mutations observed from metabolic stress (8-oxo-guanine) (386-390). Along with carcinogenesis, genomic instability is associated with diseases such as amyotrophic lateral sclerosis (AML or Lou Gherrig's Disease) and myotonic dystrophy. DNA damage is a factor that often leads to genomic instability and there are DNA repair mechanisms that handle DNA damage depending on the type and degree (386-390). MMR or mismatch repair deals with very large bulky lesions and removes large amounts of DNA. Smaller bulky adducts are removed by Nucleotide Excision Repair machinery (NER) (386-391). Smaller aberrations, especially apurinic and apyrimidinic lesion, are ameliorated by base excision repair (BER) (388-391). Some lesions cause DNA crosslinks and are so extensive that these repair mechanisms cannot remove the damage. As observed in prokaryotes, eukaryotes, including humans, have error prone DNA polymerases (i.e. DNA polymerase ζ and DNA polymerase η) which can bypass the DNA damage (392). Many lesions occur in noncoding regions and in some cases this bypass solves the issue and the mutations are silent, or ones that do not have any

genomic or phenotypic consequences. Other mechanisms, such as observed with DNA double strand breaks can be repaired by homologous recombination (i.e. recombinase Rad51) or by non-homologous end joining (NHEJ i.e. holiday junctions) (392,393). Not only can mutations occur directly within the DNA sequence but also epigenetic mutations can arise. Epigenetic regulation refers to any kind of change in DNA gene expression, regulation, or status that is independent of the primary sequence. These can include covalent changes to the DNA (.e. methylation of DNA promoters at CpG islands by DNMTs), changes in transcriptional accessibility (HATs (i.e. p300/CBP), HMTs (i.e. EZH), and HDACs (i.e. HDAC1, 6), or regulatory non-coding RNAs (i.e. lncRNAs) (383,384,394). Endogenous DNA damage by metabolic and oxidative stress is a commonly encountered by cancer cells and many transformed cells bypass this issue by having reduced expression of DNA checkpoint enzymes (i.e. CHK 1,2), enhanced DNA repair machinery or translesional bypass enzymes, or upregulation of redox enzymes (i.e. glutathione S transferases (GSTs)) (188,189,395,396).

DNA repair mechanisms and genomic instability: NER, BER, and MMR

Exposure to carcinogens does not directly result in cancer as most DNA damage is efficiently repaired by DNA repair systems, which can induce cell cycle arrest (189,383,386). DNA damage is efficiently repaired by DNA repair systems, such as nucleotide excision repair (NER), mismatch repair (MMR), and base

excision repair (BER) (Figure 25) (386-393). The former two systems deal with repairing DNA adducts. NER deals with smaller bulky lesions on DNA and uses enzymes such as excision repair cross-complementation group (ERCC 1, 4) Cockayne syndrome type A (CSA, ERCC6), Cockayne syndrome type B (CSB,ERCC8) and DNA repair protein Xeroderma Pigmentosum complementing (XPA, XPB, XPC, XPD, XPE, XPF, and XPG), which remove the lesion and through DNA polymeraseβ resynthesize the excised DNA (397-400).

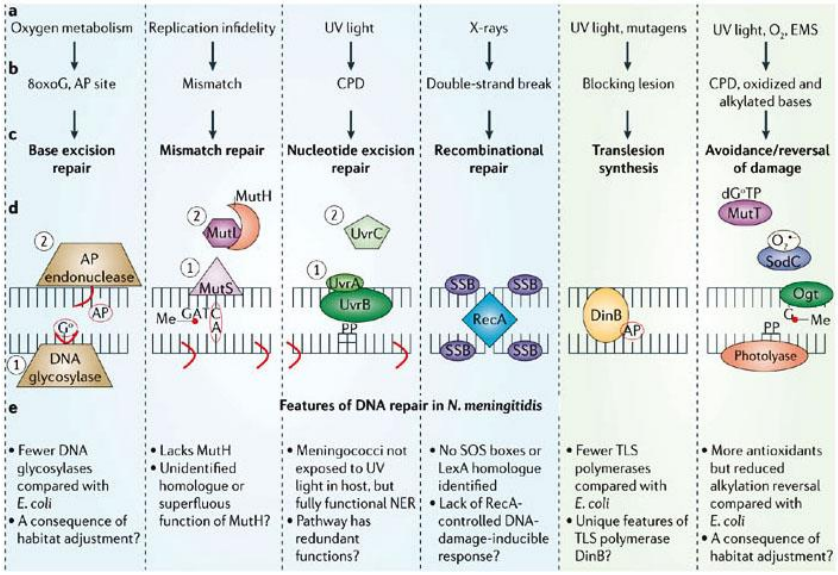

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Figure 24: DNA repair pathways. Among these are Mismatch repair (MMR), Base excision repair (BER), and nucleotide excision repair (NER). MMR repairs extensive bulky regions and resynthesizes large pieces of DNA. The BER pathway involves removal of purines and pyrimidines, generating apurinic/apyrimidinic by uridine glycosylases (OGG1 and UNG). These pathches are then resynthesized by short patch and long patch BER. NER deals with smaller bulky lesions on DNA and uses enzymes such as excision repair cross-complementation group (ERCC 1, 4) Cockayne syndrome type A (CSA, ERCC6), Cockayne syndrome type B (CSB,ERCC8) and DNA repair protein Xeroderma Pigmentosum complementing (XPA, XPB, XPC, XPD, XPE, XPF, and XPG), which remove the lesion and through DNA polymeraseβ resynthesize the excised DNA. Other pathways include translesion synthesis (with DNA polymerase η and ζ). Direct repair in is observed in bacteria such as *E. coli.*

Mutations in XP gene family members are related to the disease xeroderma pigmentosum (XP) whereas mutations in CSA and CSB are linked to Cockayne syndrome (399-401). XP is an autosomal recessive genetic disorder in which individuals are very sensitive to UV light from sunlight and are susceptible to basal cell carcinomas. Cockayne syndrome is a fatal autosomal recessive linked disorder, where individuals are very sensitive to sunlight, exhibit growth retardation, neurodegenerative disorders, eye disorders, and premature aging. Squamous cell carcinoma, melanoma, and other skin related malignancies (401,402). BER removes less bulky non helix-distorting lesions which result in generation of apurinic or apyrimidinic (AP) sites (Figure 25) (387,403). These sites are generated by DNA glycosylases such as 8-Oxoguanine glycosylase (Ogg1), 3-methyladenine glycosylase (Mag1) and Uracil-DNA glycosylase (UNG) (403). The resulting AP sites are removed my AP endonucleases (403,404). The resulting 5' and 3' ends are then processed by the end processing enzyme polynucleotide kinase phosphatase (PNKP) and DNA is synthesized by DNA polymerase β (DNA pol β) during short patch BER and DNA polymerase δ and polymerase ε during long stretch BER (403,404). The resulting 5' flap that is generated in long stretch BER is then removed by the flap endonuclease (FEN1) and the resulting nick is ligated by DNA ligase III and X-ray repair cross-complementing protein (XRCC1) (405). Mutations associated with cancer are less characterized in the BER pathway but include silencing of methyl-CpG-binding domain protein 4 (MBD4) and Endonuclease VIII-like 1 (NEIL1) (406-409).

MBD4 recognized and binds methylated CpG islands and is important in preventing transition mutations (406,407)). Specifically 5-methylcytosine can spontaneously deaminate and become thymine, which produce G:U and G:T base pairs. This leads from a G:C to an A:T transition, which is a common mutation found in 50% of p53 mutations of the tumor suppressor p53 (406,407). NEIL1 recognizes ROS-induced oxidized pyrimidines, formamidopyrimidines, and methoxy-thymine and thymine glycol stereoisomers. Hypermethylation and therefore epigenetic silencing of the NEIL1 promoter was found in 71% of HNSCC tumors and 42% of non-small cell lung cancer (NSCLC) (408). Hypermethylation is also exhibited in gastric and colorectal cancers and mutations in mutY DNA glycosylase homolog (MUTYH) (409). The gene XRCC is a rare case in which the gene is overexpressed in NSCLC and within the metastatic lymph nodes of these individuals (410).

Bulkier lesions require the MMR system and use the enzymes human MutL homolog 1 (hMlh1) and two (human postmeiotic segregation increased 1and 2 (hPms1and 2)), and human MutS homolog 2/6/3 (hMsh2/6/3), exonuclease 1 (Exo1), replication protein A (RPA), (proliferating cell nuclear antigen) PCNA, replication factor C (RFC) and DNA polymerase δ (Figure 25) (411,412). Mismatch implies that there is a non-Watson-Crick base pair present within the DNA (i.e. either not A:T nor G:C). Msh2 forms a heterodimer with Msh3 or 6 (to form hMutSα or hMutSβ respectively) and hMlh1 heterodimerizes with hMlh3, HPms1, or hPms2 (forming hMlhα, hMlhβ, and hMlhγ respectively) (411-413).

HMutS is an ATPase critical for MMR recognition of mismatches and lesions within DNA, hMlh is required for termination of mismatch excision, and its endonuclease activity requires RFC (411-413). EXO1 is an exonuclease that is involved in excision of DNA which is repaired by DNA polymerase δ and the eukaryotic version of the prokaryotic processivity β-clamp PCNA (411-413). This molecule aids in DNA polymerase attachment to the DNA and enhances its processivity (number of nucleotides per initiation event before the polymerase dissociates from the DNA) (411-413). Defects in the MMR pathway have been linked to cancer such as through microsatellite instability (414). For example, the genes *MSH2* and *MLH1* are associated with the majority of hereditary nonpolyposis colorectal cancer (HNPCC, i.e. Muir-Torre Syndrome) (415,416). The MMR related syndrome Turcot syndrome, associates familial adenomatous polyposis (FAP) with brain tumors (417). Epigenetic defects (i.e. promoter hypermethylation) in MSH2, MSH6, PMS2, and MLH1 are associated with cancer, with over 90% of these mutations in the promoter region of MLH1 (414,418). MLH1 is mutated in 73% of esophageal and stomach cancers (foveolar type), 33% of HNSCC, 69% of NSCLC, and 10% of colorectal cancer (411,415,416). Mutations in *MLH1* are most often associated with loss of other DNA repair genes (1.e *MLH3, OGG1, XRCC3, XRCC5)* (419-422).

Cell cycle deregulation and cancer

Genomic stability can also arise from losses or dismantling of cell cycle checkpoints (423-425). The cell cycle is a very tightly regulated process that

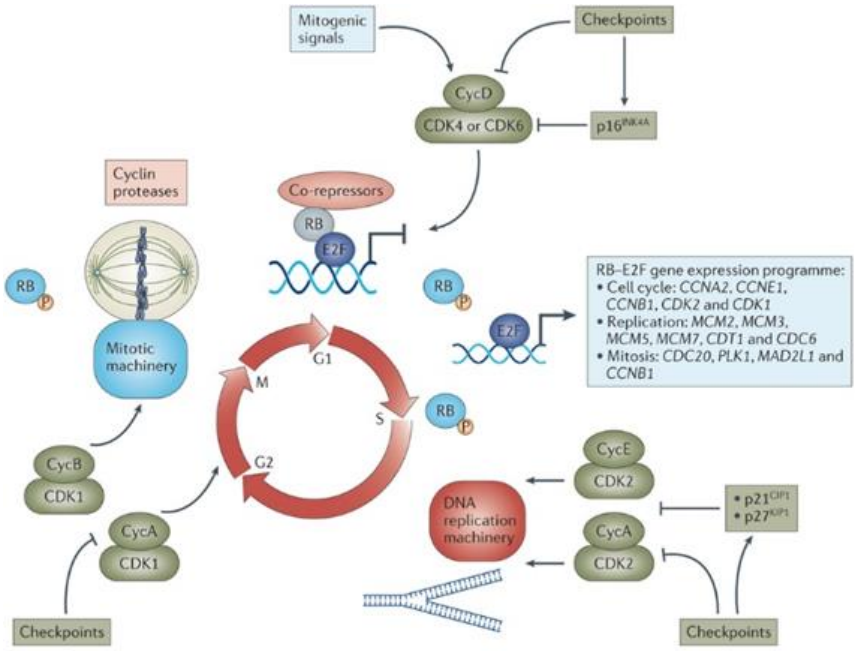

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Figure 25: The cell cycle and cancer. The cell cycle is composed of interphase and mitosis. Interphase is composed of G1, S, G2, phases of cell cycle and mitosis id composed of the M phase. At every phase of the cell cycle there are specific checkpoints which a cell must pass in order to go to the next phase of the cell cycle. If the checkpoint is not passed then tumor suppressor proteins such as p16 and p27 inhibit progression of the cell cycle by disrupting cdk/cyclin complexes (such as cdk4/6 with cyclin D1). Cancer cell will epigenetical suppress tumor suppressor proteins like p16 and p27.

contains several sentinels that assure specific cellular and molecular criteria are met before a cell can successfully progress to another stage of the cell cycle (Figure 26) (423-425). The cell cycle is developed into three major phases: interphase, mitosis, and cytokinesis. Interphase has three major phases, which can be divided into G1, S, and G2/M (423-425). There are cellular checkpoints that are installed between each border of these phases and certain criteria must be met in a normal cell in order to gain entry into the next phase of the cell cycle. In cancer cells these checkpoints are lost or dismantled, resulting in controlled cell

cycle progression and initiation. This is often associated with accumulated DNA damage and genomic instability (423-425). G1 is the DNA replication preparatory stage, S phase involves DNA replication, and G2/M phase involves preparation into mitosis and is instigated by mitotic promoting factor (MPF) (424,425).

Progression of the cell cycle is strictly regulated by proteins known as cyclins. Originally discovered by Timothy Hunt (along with Paul Nurse and Leeland Hartwell), these proteins have cycles of expression to where they are induced and then subsequently degraded (423,426). The cell cycle has gradual fluctuations in these cyclins, with an increase in one cyclin that is proceeded by waning expression of another cyclin. Expression of cyclins flows as D, E, A and B cyclin respectively (426). These cyclins are important for activation of cell cycle kinases that promote cell cycle progression known as cyclin dependent kinases (CDKs) (426). They not only promote the cell cycle but also prevent precocious initiation of another cell cycle before the preceding cell cycle has been completed. CDKs are a family of serine/threonine protein kinases that are associated and activated at specific stages of the cell cycle (426). By themselves, they have very low intrinsic catalytic activity and require association of their respective positive regulatory cyclin. Whereas cyclin levels are ephemeral and have dynamic expression and stability, CDK expression is constant and stable (426,427). Once a CDK is associated with a cyclin, it requires activation by a phosphorylation by the enzyme CAK (cdk activating protein). Cells can initiate interphase and become quiescent, entering the so-called G0 phase and in certain cases stay

there indefinitely (423-425). This is different from a senescent cell as unlike cellular senescence, quiescent cells can be stimulated by mitogenic signals and their replicative silence is reversible. When these cells are stimulated by mitogens, CDK4 and CDK6 associate with D cyclins (cyclin D1, D2, and D3 and association of CDK4/6 with cyclin D is vital for progression through the G1 phase and is negatively regulated by Rb, p16, and p53/p21 signaling (423-427). Rb the retinoblastoma protein and the first tumor suppressor characterized in the childhood cancer retinoblastoma is a negative regulator of the cell cycle and is a sentinel of the G1/S checkpoint (177,178,423-426). Excessive DNA damage can lead to upregulation of p53, which can stimulate p21 and inhibit the cell cycle (188, 427,428). The cycle cycle can also be arrested by p16 and p14, with the former binding to CDK4/6 and inhibiting association with cyclin D. With mitogenic stimuli, Rb is hyperphosphorylated, resulting in liberation of the transcription factor E2F, which induces expression of cyclin E and cyclin A (177,178,188,423-425). The induction of these cyclins is associated with the subsequent activation of CDK 2 and this promotes DNA replication. After successful DNA replication, cyclin B is induced and this leads to activation of CDK1 and the entry into mitosis. In G2 phase, CDK1 is kept inactive until the end of G2 and entry into M phase by the inhibitory protein Wee1 kinase (423-426). Wee1 phosphorylates T14 and Y15 of CDK1 and this is reversed by the cognate phosphatase CDC25, which stimulates CDK1 activity and entry into the M cycle (423-426). The activation status of CDK is also negatively regulated by phosphorylation in the ATP binding pocket, which

is vital for providing the phosphate for phosphorylation because dephosphorylation of ATP is thermodynamically favorable and permits otherwise thermodynamically unfavorable phosphorylation reactions (426,427). Specific residues within the DNA binding pocket can also be phosphorylated and this can be reversed by specific phosphatases. Proteins known as cyclin dependent kinase inhibitors (CKI) include p16 and p14ARF as well as p15 p18, and p19 and these are mainly involved in G1/S regulation (187-190, 427,428). This checkpoint has the highest level of regulatory involvement due to the fact this is a "commitment" checkpoint. This means that as soon as a cell goes beyond this checkpoint it is committed to completing the cell cycle and if it fails to do so it will undergo apoptosis. CDKs can also be regulated at the level of subcellular location since their nuclear import is critical to their function (429,430). For example, the cdk1/cyclin B complex is sequestered in the cytosol to prevent premature phosphorylation events leading to entrance into mitosis (429). Other proteins that are involved include the phosphatase cdc25, which is sequestered by 14-3-3 in the cytosol. DNA replicative fidelity and proficiency are vital to prevent genetic instability (430).

Checkpoint kinases (CHK1 and CHK2), ATM, and ATR

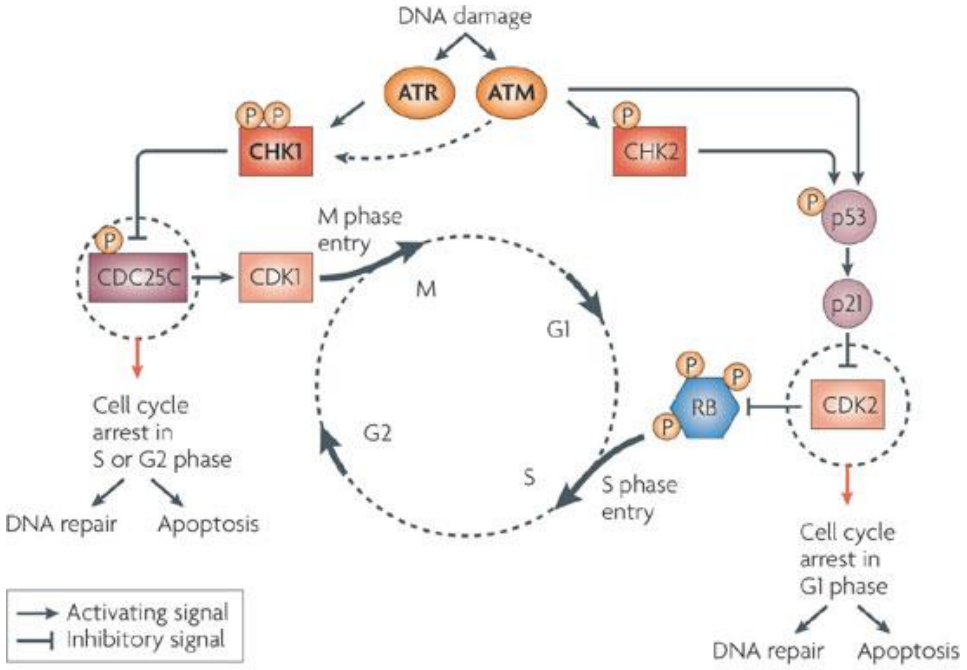

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Figure 26: Checkpoint kinases. The cell cycle checkpoints are regulated by kinases known as checkpoint kinases.. Upon DNA damage, the kinases ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia Rad 3 related) phosphorylate checkpoint kinases (CHK) CHK 2 and 1 respectively. CHK1 phosphorylates CDC25C phosphatase and inactivates it. CDC25C reverses the phosphorylation of Wee1 kinase on cdc2 (CDK1) on two phosphorylation sites T14 and Y15. CHK2 phosphorylates p53 and activates p21. This inactivataes CDK2 and promotes cell cell arrest, DNA repair, and apoptosis.

DNA damage can be detected resulting in activation of the kinases ATM and ATR, these can stimulate checkpoint kinase CHK1 and CHK2 (Figure 27) (187-189, 427,428,431). CHK1 is one of the main components of the DNA damage checkpoint pathways and it preserves DNA integrity. CHK1 is activated by ATM/ATR (at S317 and S345), activated CHK1 phosphorylates cdc25, and inactivates it (187-189, 431). ATR also depends upon the action of claspin, BRCA1, and TOBP1. It has a dual role in this mode of phosphorylation at both the

G1/S and the S phase checkpoints by phosphorylating CDC25A at the G1/S checkpoint and then CDC25A and CDC25C at S and G2/M checkpoints (187-189, 431-433). Phosphorylation of CDC25A leads to its proteasome-dependent degradation whereas phosphorylation of CDC25C at S216 leads to 14-3-3 association and its cytoplasmic sequestration (187-189, 431-433). DNA damage causes single strand breaks, which is bound by several units of RPA (replication protein A) which prevent its degradation by cellular exonucleases (387,390). RPA recruits recombinase protein Rad17 and the ATR/ATRIP complex, which further activates CHK1 (391, 433,434). ATM activates CHK2 and is recruited to double strand breaks by the MRN complex, which consists of Mre11, Rad50, and Nbs1. Along with assisting ATM mediated CHK2 activation it is involved in double strand break priming for homologous recombination and NHEJ (431-434). CHK1 and wee kinase inhibitors have been considered as potential chemotherapeutic targets, as adjuvants in chemotherapies that contain DNA damaging agents such as doxorubicin, cyclophosphamide, and others. For example, the pyeazolo-pyrimidine compound MK-1775 has been used as an inhibitor of Wee1 and is in phase II clinical trials for ovarian cancer with combinatorial paclitaxel, carboplatin treatment (435). The drug UCN 01 has been developed as a CHK1 inhibitor however has had limited success due to lack of efficacy, poor pharmacokinetics, and enzymatic promiscuity (436).

Maturation promoting factor (MPF) and anaphase promoting complex (APC)

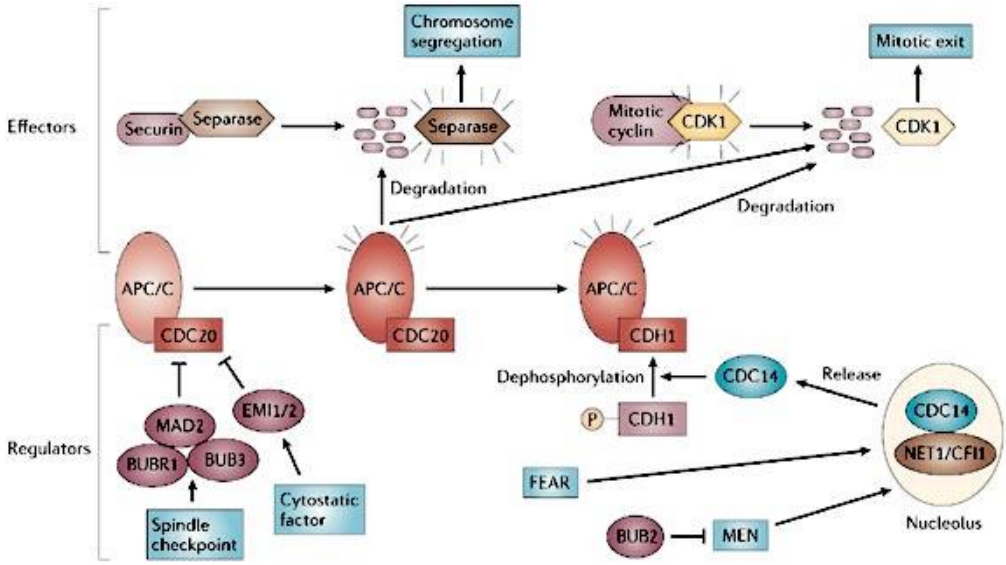

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Figure 27: MPF and APC. The early stage of mitosis involves the kinase activity of mitotic promoting factor (MPF), which is a complex of CDK1 and cyclin B. MPF is then phosphorylated at T161. As the cell cycle progess towards metaphase and the chromosomes are aligned at the metaphase plate, anaphase promoting complex (APC) poly-ubiquitinates cyclin B and is then subsequently degraded via the proteasome. APC ubiquitinates securing and frees seperase, which is required for completing of anaphase. APC is also regulated by MAD2 (mitotic arrest deficient 2), BUBR1 (BUB related protein 1), BUB3 (mitotic checkpoint protein BUB3), and EMI 1/2 (Emilin 1/2). In late M phase, APC exchanges its activator CDC20 for CDH1, which is mediated by CDC14, a phosphatase. Most of cell cycle cdc14 is sequestered in the nucleolus and is freed by MEN (mitotic exchange network) and FEAR (fourteen early phase anaphase). Once chromsomes arealigned at metaphase plate they are separted by active separase, and chromatids are pulled to opposite poles located at centrosomes by kinetochore microtubules. Aberrant APC leads to chromosomal instability in cancer

After successful completion of interphase, cells enter mitosis, which is promoted, by MPF or maturation promoting factor (Figure 27) (435-437). Mitosis is the duplication of chromosomes and this duplication requires successful duplication that is followed by complete and successful segregation of chromosomes. Mitosis is broken down into prophase, prometaphase, metaphase,

anaphase and telophase. MPF promotes mitosis all the way up to metaphase where chromosomes that have been duplicated are aligned at the center of the metaphase plate (423-425, 430). In order for successful segregation of the chromosomes MPF must be degraded and this is in part accomplished by a multisubunit protein complex known as the anaphase promoting complex (APC, cyclosome), and the other SCF (Skp1/CUL1/F-box protein) (437). These are two E3-ligase complexes that promote entry of cells into anaphase by targeting MPF for proteasome-dependent degradation. APC consists of 11 subunits that contain WD-40 containing adaptor proteins, cdc20, and Hct1 to recognize and bind to its substrates. There are also two different forms of APC: APC^{cdc20} and APC^{cdh1}, with the latter being essential for exiting metaphase and the other form playing more of a role at G1/s transition. APC recognizes potential substrates by KEN box and destruction box motifs (RXXLXXXXN/D/E) (438). APC also ubiquitinates cyclin B and securing, targeting them for proteasome-dependent degradation. APC is tightly regulated and to prevent chromosomal instability and premature separation of chromosomes by negative regulation mediated by Mad2 and BubR1, which is part of the spindle assembly checkpoint (438,439). When APC is activated it leads to dissociation of securin from seperase and its proteasome-dependent degradation (Figure 30) (438,439). This is essential for separation of sister chromatids at the metaphase plate and completion of anaphase (423-430, 437-439).

Microtubules and microtubule associated proteins (MAPs) and cancer

When cells undergo mitosis, they have not only duplication of chromosomes but also microtubule organizers and mitotic spindle machinery, and complexes known as centrosomes are at the focal point of these structures (423-425, 440). Microtubules are one of the three main types of cytoskeleton proteins (the others being microfilaments and intermediate filaments, and the lesser-known septin proteins) (440,441). Microtubules are approximately 25 nm in diameter and consist of 13 protofilaments that arrange into a hollow tube. These protofilaments are composed of alternating αβ tubulin heterodimers. They assemble through association with GTP, which promotes microtubule polymerization. Hydrolysis of GTP leads to rapid depolymerization and this is a critical feature in regulation of microtubules through dynamic instability (440-442).

In cells undergoing mitosis there are two centrosomes, each with pair of centrioles that are arranged orthogonally with respect to one another. These contain a multitude of proteins that are important for centrosomal assembly and function. Its activity is also dependent on the action of kinases, namely polo like kinases such as PLK-4, which is indispensable for centrosomal activity and its inactivation leads to cell cycle arrest at metaphase. Centrosomes have microtubule organizing centers (MOCs), which have microtubules organizing and emanating from γ-tubulin ring complexes (γ-TURCs) (440-443). These microtubules emanate and form astral microtubules and kineticore microtubules,

with the latter extending and making direct contacts with chromosomes at their kineticore structures. Microtubules can move chromosomes through kinetochore structures to the metaphase plates and subsequently segregate them through anaphase (423-425, 440-443).

Upon segregation of chromosomes the microtubules depolymerize and pull apart sister chromatids. There are also motors associated with microtubules, such as minus end (towards the cell center) dyneins and plus end (toward the cell leading edge) kinesins (with kinesin 11 being an exception) (443,444). These proteins transport protein and cargo along microtubules and are involved in cell cycle progression (443,444). Other microtubule associated proteins such as Tau, XMAP215, Lis1, EB1, EB2, and Dynamatin, stabilize microtubules through phosphorylation (443-445). As a result, microtubules are a target for cancer chemotherapy. Drugs such as vinca alkaloids vincristine, vinblastine and colchicine drugs like colchicine, and combrestin destabilize microtubules and prevent polymerization (440-445). Dynamic instability is crucial for cell cycle progression and drugs that have the opposite effect such as taxane-derived drugs like Taxol and docetaxel prevent microtubule depolymerization

Aurora A kinase

Another group of proteins involved in chromosome segregation are the aurora kinase family. This consists of aurora kinase A and aurora kinase B (446). Aurora A functions during prophase and is required for centrosomal function

(Figure 28) (446). Aurora B is involved in mitotic spindle interaction with the centromere of chromosomes through their kinetochores (446,447). Aurora A localizes by centrosomes during G1/S transition and then associates with mitotic spindles during later phases of mitosis (446,447). It is critical for the formation of the mitotic spindle and phosphorylates many proteins involved in its formation, including TACC and kinesin-5 and it also promotes the formations of γ-TURCs (448). Aurora A ensures proper chromosomal alignment along the metaphase plate during prometaphase, and exit of mitosis during telophase. Aurora A kinase is often implicated in cancer progression and has been shown to be instrumental in EMT in neuroendocrine tumors and prostate cancer (449). Overexpression of Aurora A can lead to aneuploidy, especially in cells that are deficient of p53 activity, which regulates Aurora A.

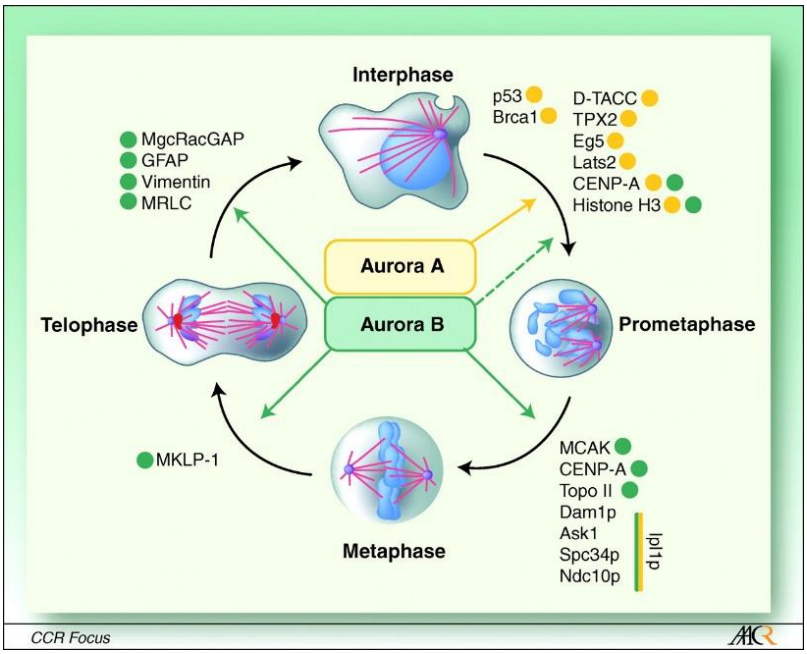

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Figure 28: Aurora kinases. Aurora kinases and their substrates during various stages of the cell cycle. The colored circles denote known substrates of Aurora A and B across the cell cycle. Aurora A negatively regulates BRCA1, p53 and Lats, while positively regulating TACC, CENPA, and Eg5. Tpx is also phosphorylated by aurora A, and is a critical component requires for associating aurora A to spindle microtubules and its autophosphorylation. GOLGA2 interacts with importin α, the negative regulator of tpx2, and enables Tpx2 to associate with aurora A and promotes microtubule nucleation. Aurora B phosphorylates MCAK (microtubule centrome associated kinesin), MRLC (myosin regulatory light chain), MKLP-1 (mitotic kinesin-like protein 1), all of which are essential for microtubule activity and increased paclitaxel resistance in cancer. Ask1 (apoptotic signaling kinase 1), Spc34p, and Ndc10p (centromere binding factor) are substrates of phospholipase LPL1. Borealin interacts with INCENP, survivin, and aurora B kinase to promote the cell

Aurora B kinase

Aurora B kinase expression is maximal at G2/M transition and associates with chromosomes during prophase and is involved in the chromosomal passenger complex, a critical complex involved in cell cycle progression, which consists of itself, survivin, Borealin, HBX, and INCENP. Surivin is an IAP protein discussed previously and INCENP is an inner centromere protein (446,447,450). CENPs (or centromere proteins) describes a class of proteins which are divided

into constitutively binding and passively (or passenger) chromosomal binding proteins (Figure 28) (446,447,450). Passenger proteins are those that localize to centromeres during metaphase to anaphase transition and include CENP (centromere protein E), Kinesin-like protein KIF2c, kinesin family member 22, Dynein DYNC1H1, CLIP1 (CAP-GLY domain containing linker protein), and CENPF (450-452). These localize to centromeres during metaphase-anaphase and promote chromosomal passenger complex activity. INCENP regulates the catalytic activity of Aurora B along with survivin and Borealin (450-452). In order for Aurora B to localize to the centromeres during metaphase the protein CENP-A needs to be phosphorylated and promotes assembly of the kinetochore (450-452). This phosphorylation occurs at S7 on CENP-A and is mediated by Aurora A.

Aurora B also associates with MAP EB1 during anaphase and its overexpression increases Aurora B catalytic activity and blocks the catalytic activity of the negative counterpart of Aurora B protein phosphatase 2A (450-452). Aurora B is also important in the orientation of the chromosomes and ensures interaction with chromosome kinetochores and kinetochore microtubules (450-452). Aurora B mediates phosphorylation of Histone H3 on S10 and promotes chromosomal condensation and adhesion (452,453). The spindle assembly checkpoint inhibits the progression of mitosis from metaphase to anaphase and one of the criterion is the proper biorientation of sister chromatids and deficiency of Aurora B leads to aberrant progression of the cell cycle and has implications in

cancer (452-454). Aurora B also localizes the negative regulators of APC/C, MAD2 (mitotic arrest deficient 2) and BubR1 (454). When misaligned chromosomes are presented at the metaphase plate this protein is involved in preventing sister chromatid segregation. It binds to Mad1, which binds to cdc20 and inhibits APC. Abnormal levels of Aurora B lead to aneuploidy and this has led to the development of drugs that target this kinase (452-454). The drug BI811283 developed by Boehringer Ingelheim for patients with AML is an Aurora B kinase inhibitor that binds to the ATP binding pocket of Aurora B (455). This leads to polyploidy that is eventually too unstable to maintain and leads to cell death. The drug is also in phase I and 2 clinical trials for NSCLC, brain cancer, head and neck squamous cell cancer, CRC, and ovarian cancer. BI811283 has also met with issues that limit its use as a chemotherapeutic agent due to development of leucopenia and neutropenia (456).

Chromosomal translocations, HPV, and genomic instability

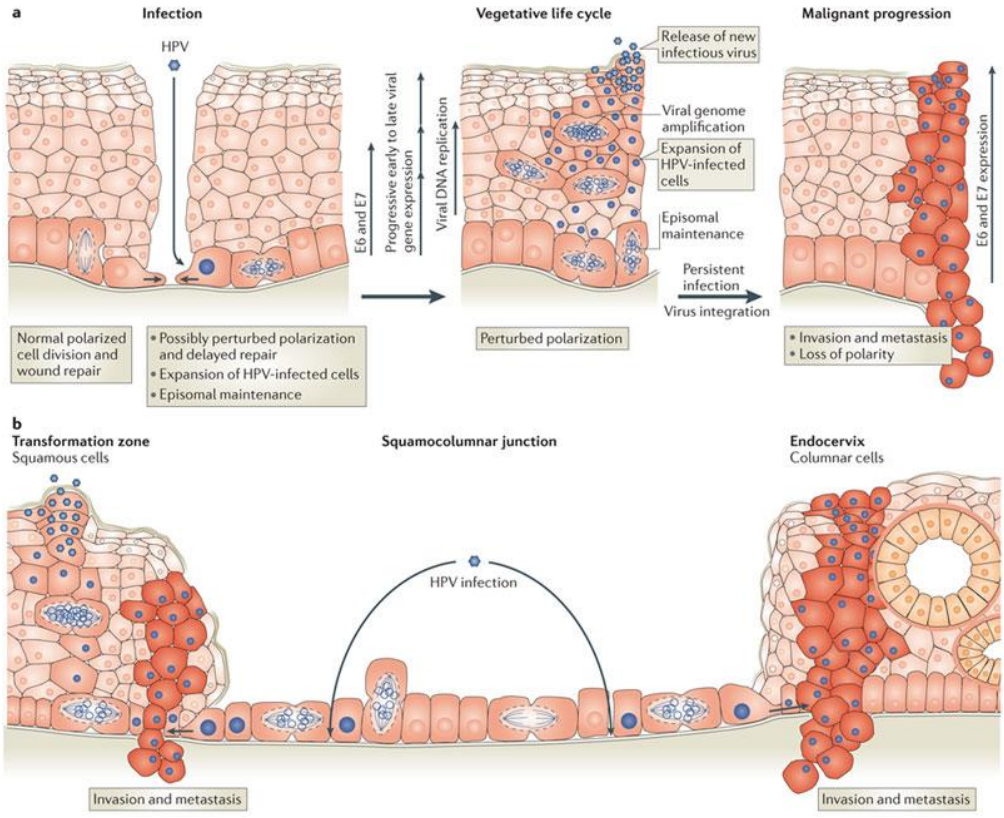

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Figure 29: Human papilloma virus (HPV). Lifecycle and stages of HPV (human papilloma virus) and how HPV induces cancer, such as cervical cancer.

Chromosomal instability is also associated with chromosomal translocations. This can be spontaneous (as seen in CML) or a result of oncoviruses (33,34,457). As stated previously CML is the result of the Philadelphia chromosome, which results in the BCR-ABL fusion gene products, which can be inhibited by Gleevec (imatinib) (31,32). Retroviruses can cause cancer in mammals, although this is not as prevalent in primates, including

humans. This however does not preclude the existence of virus-derived malignancies. One example is the development of Burkett's Lymphoma from the Epstein - Barr virus (EBV), Kaposi's sarcoma from HIV, and HCC from HBV (33,34,457). Another virally induced cancer is cervical cancer from the human papilloma virus (HPV) (Figure 29).

HPV is a double stranded DNA virus with 100 different subtypes, however, only high-risk mucosotropic (HPV-16 and HPV-18) are causative agents of cancer. Low risk type HPV only results in benign lesions such as genital warts (457,458). High-risk HPV has a long latency period and encodes two oncogenes, E6 and E7, which facilitate immortalization and transformation of cervical epithelium by a series of steps. Alone the proteins do not confer a transformed phenotype and just induce replicative immortality(stimulate telomerase) and promote other hallmarks of cancer such as unstained proliferation, evasion of tumor suppressors and apoptotic signals, and most importantly induce chromosomal abnormalities (457,458). This can confer a replicative advantage and produce clonally selected transformed cells. E6 and E7 degrade the RB protein, as well as inactivate p21 and p27 to promote sustained E2F signaling and activation of CDK2 through cyclins A and E. This can activate a negative feedback loops as sustained E2F activates $p14^{ARF}$, which in normal cells serves as an autoregulatory loop on E2F and CDK activity, as this activates p53 (457,458). To circumvent this E6 activates E6-AP (a HECT-type E3 ubiquitin ligase) which targets p53 for proteasome-dependent degradation. HPV E7 can also promote

the supernumerary of centrosomes and mitotic spindles, causing asymmetric chromosomal segregation and result in aneuploidal cells, which seems to be dependent on aberrant CDK2 activity induced by E7. E6 can induce polynuclear or multilobulated nuclei as the result of inhibition of cytokinesis after persistent mitotic rounds (457,458). High-risk HPV induced cervical cancers frequently have abnormal chromosomal structures and aneuploidy. E6 and E7 can induce anaphase bridges as the result of the generation of unprotected chromosomal ends as the result of DNA damage or telomere erosion (457,458). HPV positive cells are susceptible to this type damage as p53-induced DNA repair mechanisms are abolished to promote HPV induced carcinogenesis. High-risk HPV-16 cells have higher levels of phosphorylated H2AX, which is a marker for DNA damage, and recruits the DNA repair machinery (457,458). HPV is a major cause of cancer-induced mortality in females and much research has gone into elucidating HPV mechanisms of carcinogenesis. Currently there are three different FDA approved vaccines for HPV, Gardasil, Gardasil 9, and Cervarix, which are all efficacious against HPV-16 and HPV-18 (457,458).

Tumor promoting inflammation

Cancer and inflammation

Chronic inflammation has been linked to enhanced tumorigenesis and this idea was initially postulated in 1863 by Rudolf Virchow (459). Virchow contributed much of the early knowledge of cancer and first described the origination of cancer

from previously normal cells. He observed that there was a correlation between certain cancers and an increase in leukocyte number that produced chronic inflammation (458). He was the first to characterize leukemia, chordoma (a cancer associated with remnants of the rudimentary notochord) and make the case for a specific link between inflammation and cancer (458). Indeed many cancers are associated with inflammation such as mesothelioma (inflammation associated with asbestos exposure), prostate (chronic benign prostatic hyperplasia) bladder cancer (sustained urinary tract infections), pancreatic (pancreatitis), cervical cancer, CRC (colitis, IBD), melanoma (excessive UV exposure, exposure to irritants), and esophageal (acid-reflux diseases) (459-465). The experimental evidence that demonstrates how steroidal drugs (i.e. prednisone, dexamethasone) and non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, sulindac sulfide, and ibuprofen attenuate cancer cell proliferation and progression also corroborates this concept (459-465). During injury, immunological activation, or pathogenic infection produces inflammatory cells such as macrophages release inflammatory cytokines such as TNF-α and proliferative chemicals that potentiate neoplastic propensity (466).

Inflammation is the body's response to injury and it is mediated by leukocytes, consisting of neutrophils, monocytes (which extravasate into wounded tissue and differentiate into macrophages, and eosinophils, which trigger mast cells to release histamine and promote and inflammatory/allergic response (466). Neutrophils use a four-step mechanism that involves "a rolling stop" that

coordinates recruitment of other inflammatory cells to the provisional ECM, enabling fibroblast and endothelial cells to proliferate and migrate (467). Steps include activation of the selectin family of adhesion molecules (L-selectin, P-selectin, and E-selectin), which facilitate the “rolling” along the vascular endothelium (466,467). Neutrophils upregulate integrins (α4β1 α4β7) which enable binding to vascular cell-adhesion molecule-1 (VCAM-1) and MadCAM-1 respectively. MMPs are then activated to create a route of entry for the neutrophil to the site of injury (466,467). Platelet aggregation which is followed by the multistep coagulation cascade with the aid of phylloquinone and menaquinone (vitamins K1 and Vitamins K2 respectively), provide proteins that promote inflammation, including heparin, thrombin, serotonin, fibronectin and von Willebrand factor, PDGF, TGFα, and arachidonic acid (466-468).

Cyclooxygenases (COX) and chemokines

Arachidonic acid is the precursor to a group of lipid signaling molecules called prostaglandins (466). Cyclooxygenases (COX) enzymes, which convert arachidonic acid into PGE (COX-2), and other pro-inflammatory and pain mediators synthesize these molecules. Aspirin and NSAIDS inhibit these enzymes (459-465). Formation of granulation tissue is mediated by chemotaxis of neutrophils, monocytes and fibroblasts. Neutrophils produce early response inflammatory cytokines such as TNF-α, lL-1α and IL-1β (466,467). Monocytes, which become macrophages within the injured tissue, are recruited by PDGF, PF-4, chemoattractant protein-1, -2, CCl2, CCl8, CCl7, CCL3, CCl4, and the above-

mentioned cytokines (466-469). These macrophages produce a profuse amount of growth factors (TGFα, TGF-β, PDGF, bFGF) that promote tissue repair and promote inflammation (466-469). Chemokines attract specific leukocyte populations, which produce many inflammatory and wound healing cytokines, such as TNFα and TGFβ1 respectively, with the latter promoting type II EMT that is associated with wound healing. TGFβ1 can negatively regulate the inflammatory response in cancer cells and this mode of regulation is abrogated (466-469). Neutrophils and eosinophils are first recruited to an injured site and this leads to recruitment of mast cells, which release histamine and highly sulphated proteoglycans and proteases, and resulting in recruitment and differentiation of monocytes into macrophages. Macrophages often promote inflammation as a normal response to pathogenic infection, allergy, or other foreign insults (467,469).

Cancer and the immune system

Tumor cells take advantage of this leukocyte attraction mechanism and produce their own chemokines to attract leukocytes, which may be composed of a heterogeneous population of neutrophils, eosinophils, mast cells, dendritic cells and macrophage, and lymphocytes (i.e. NK cells, T-cells, and B-cells) (465-469). This population can produce a concoction of cytokines, ROS inducing species and mediators, serines and cysteine proteases, MMPs, membrane perforating agents (i.e. granzyme from neutrophils), interleukins, and interferons (470).

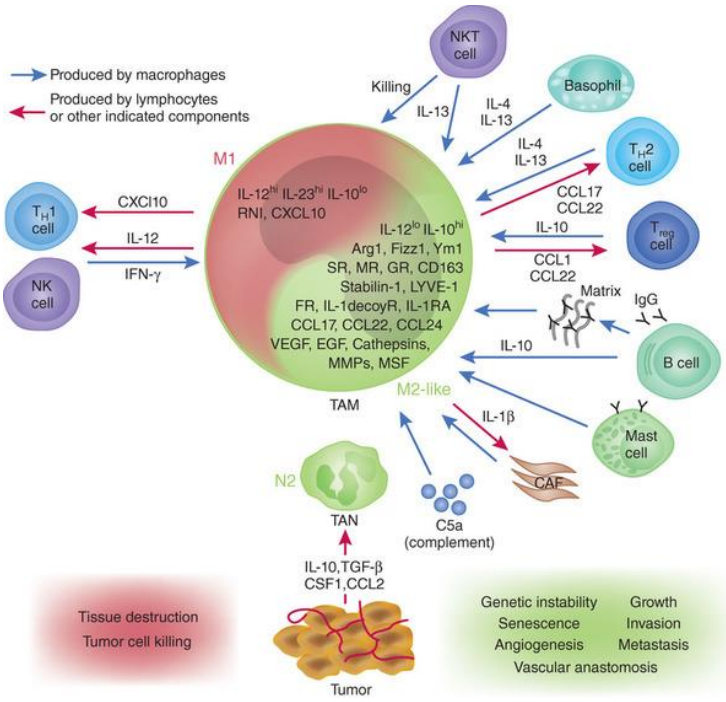

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Figure 30: Tumor associated macrophages. Tumor associated macrophages are associated with enhanced carcinogenesis. Monocytes are recruited and extravasate tissue by chemokines CCL2, M-CSF (macrophage-conlony stimulating factor), and VEGF. These monocytes differentiate into macrophages by IL-3 and M-CSF. These macrophages then become M2-polarized by other CSFs, IL-4, IL-10, and, TGFβ. These TAMS not only promote tumor growth (by production of MMPs, VEGF, PDGF, FGF, and TGFβ) and metastasis (IL-1β, MMPs, and TNFα) but suppress cytotoxic T-cell responses by production of IL-10, and stimulate production of T regulatory cells (CCL17, CCL22). T cells also become anergic by secretion of CCL 18. TAMS have a profound effect on the tumor microenvironment and serve as garrisons for tumor growth.

Monocytes differentiate into immature dendritic cells in the presence of IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) (465-470). Dendritic cells are antigen-presenting cells (APCs) which collect antigens from sites of injury and infection, present them to T-cells, and stimulate their clonal proliferation within lymph nodes. Tumor cells produce IL-6 and CSF-1 to promote monocyte differentiation into macrophages (470). Tumor associated macrophages (TAMs) are highly implicated in inflammation induced

carcinogenesis and are recruited by monocyte chemotactic protein (MCP) chemokines (Figure 30) (470,471). TAMs produce angiogenic and lymphangiogeneic growth factors and proteases, which potentiate carcinogenesis. Moreover TAMs produce anti-cytotoxic T-cell interleukin, IL-10, which attenuates $CD8^+$ T cell mediated response, even when TAMs are eradicating tumor cells (470-472). Several types of cancer take advantage of this. For example, melanoma, a very aggressive type of cancer, in response to macrophage activation express IL-8 and VEGF-A and induce angiogenesis (470-472). These TAMs express VEGF-C and VEGF-D, which as discussed previously are potent cognate ligands for VEGFR-3, which stimulates lymphangiogenesis, and potentiates lymph node infiltration and metastasis.

Ulcerative colitis and irritable bowel disease (IBD)

As stated earlier many infectious diseases are associated with chronic inflammation and cancer. Ulcerative colitis and IBD have demonstrated one of the strongest cases for such as relationship (473). As discussed in the genomic instability section, HPV is associated with cervical cancer, HBV with liver carcinoma, and prolonged schistosomiasis from the trematodes *Schistosoma* are associated with CRC (473,474). Stomach cancer (second most common cancer worldwide), and ulcers which increases the propensity for gastric cancer are caused by *Helicobacter pylori* infection. Infected individuals will have macrophages that express migration inhibitory factor (MIF), which suppresses p53 activity and enhances tumorigenesis (475). With persistent infections and

inflammatory conditions, leukocytes induce DNA damage in these proliferating cells by generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Repeated tissue damage and repair by the tumor results in the generation of cells with genomic alterations that can lead to genomic instability (473-475). This along with expanding the lifespan of macrophages and other immune cells creates a very opportunistic environment for tumor cells to exploit; this is in conjunction with cells that already have acquired successive DNA damage and gain of function cell proliferative mutations (476). Tumor cells can use autocrine production of cytokines and chemokines to perpetuate their growth and progression. Melanoma cells produce CXCL1, CXCL2, CXCL3, and CXCL8 promote autocrine-stimulated cell proliferation (477). Ligands to CXCR2 have also been linked to the autocrine growth of pancreatic, head and neck cancer, and NSCLC. Macrophage inflammatory chemokine-3α (MIP-3α) is overexpressed in pancreatic carcinoma cells and tumor infiltrating macrophages (TIMs). This protein enhances TAM migration and the growth of neoplastic cells simultaneously. Certain chemokines can also induce angiogenesis, such as those with the ELR amino acid sequence N-terminal with respect to the CXC motif have potent angiogenic stimulating potential by stimulating endothelial cell migration in a chemotactic manner. Lack of this ELR motif has antagonistic activity and inhibits angiogenesis (476). ELR+ chemokines bind to CXCR2 and CXCR1, whereas those that lack the ELR sequence bind to CXCR3, CXCR4, and CXCR5. Stromal cell-derived factor (also known as CXCL12) induces angiogenic

expression of VEGF-A which upregulates CXCR4 on endothelial cells. CXCL12 has also been linked to metastasis and triggers chemotaxis of malignant mammary carcinoma cells (476). This demonstrates the direct role inflammation can have on tumor physiology by enhancing angiogenesis by chemokine production as well as metastasis.

NSAIDS as anticancer agents

NSAIDs exhibit anticancer activity in colorectal, lung, esophageal, and gastric cancer. NSAIDs (like aspirin, ibuprofen) inhibit cyclooxygenases (i.e. COX-1 and COX-2) and prevent conversion of arachidonic acid into prostaglandins, which mediate pain and inflammatory responses (459-465). Aspirin (acetylsalicylic acid) acetylates and inactivates COX-1 and COX-2, and inhibits the synthesis of PGE, endoperoxides, and thromboxane A2. Some NSAIDs have anti-inflammatory/antitumorigenic activities that are independent of COX inactivation (459-465). Tolfenamic acid induces the downregulation of Sp proteins which attenuate cancer growth (478). Some NSAIDS may induce apoptosis by directly targeting mitochondria to induce cytochrome c release and induction of caspase 3 (459-465,478).

Cancer and the inflammasome

Another complex that has been implicated in tumor progression, though in a somewhat equivocal way, is the inflammasome (Figure 31) (479). The inflammasome is a component of the innate immune system and consists of

caspase 1, caspase 5, PYCARD (PYD and CARD containing domain), and NALP (Nod-like receptor) (479,480). PYCARD consists of two protein domains, a pyrin domain (PYD) and a CARD domain. The pyrin domain is a class of death fold proteins that enable interaction with proteins that have the same domain and CARD domains enable caspase recruitment (see evasion of apoptosis section) (479,480).

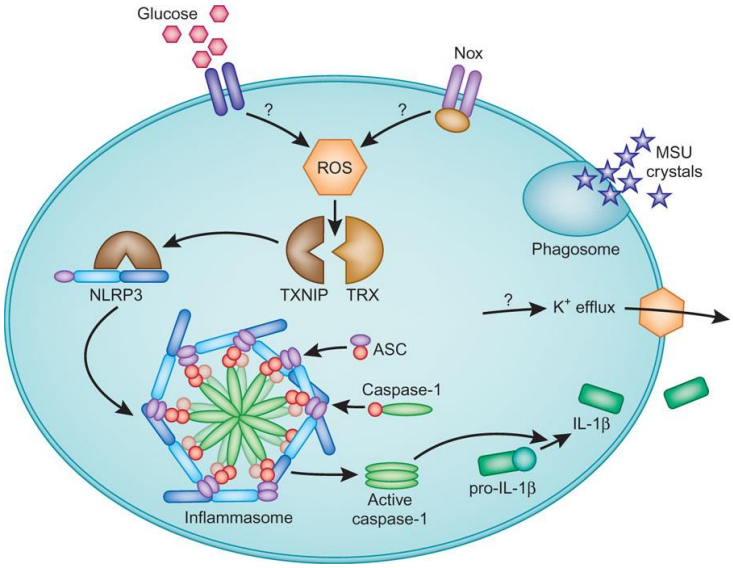

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Figure 31: The inflammasome. The inflammasome is a component of the innate immune system and consists of caspase 1, caspase 5, PYCARD (PYD and CARD containing domain), and NALP (Nod-like receptor). PYCARD consists of two protein domains, a pyrin domain (PYD) and a CARD domain. The pyrin domain is a class of death fold proteins that enable interaction with proteins that have the same domain and CARD domains enable caspase recruitment (see evasion of apoptosis section). NALP proteins detect pathogenic epitopes such as peptidoglycans that stimulate potassium efflux and activate caspase 1. NOD-like receptors are part of a large family of receptors known as pattern recognition receptors (PRRs). NLRP1 can stimulate the formation of the inflammasome and activate caspase 1, which leads to the formation of IL-1β and IL-18. This stimulates production of IFN-γ, NK cell activation, and proteolytic inactivation of IL-33 (Th2 responsive cytokine). Production of IL-1β and IL-18 has also been implicated in the proliferation and metastasis of neoplastic cells. Particular SNPs in seven inflammasome genes (NLRP1, 3, 6, CARD8, IL18, IL1β, and TNFAIP3) were linked with increased protection against HPV-induced cervical carcinoma. The inflammasome is also implicated in promoting asbestos-induced mesothelioma.

NALP proteins detect pathogenic epitopes such as peptidoglycans that stimulate potassium efflux and activate caspase 1 (481). NOD-like receptors are part of a large family of receptors known as pattern recognition receptors (PRRs) (481,482). NLRP1 can stimulate the formation of the inflammasome and activate caspase 1, which leads to the formation of IL-1β and IL-18 (481,482). This stimulates production of IFN-γ, NK cell activation, and proteolytic inactivation of IL-33 (Th2 responsive cytokine). Production of IL-1β and IL-18 has also been implicated in the proliferation and metastasis of neoplastic cells (466,467). Specific SNPs in seven inflammasome genes (NLRP1, 3, 6, CARD8, IL18, IL1β, and TNFAIP3) were linked with increased protection against HPV-induced cervical carcinoma (480-482). The inflammasome is also implicated in promoting asbestos-induced mesothelioma (483). Dietary components such as cholesterol can serve as a promoter in colorectal cancer when used in azoxymethane treated mice and this was shown to be through an inflammasome-dependent pathway (484). Though not all the details are explicitly clear, inflammation plays a role in tumor promotion, angiogenesis, and metastasis, through leukocyte infiltration, innate immune responses, or autocrine signaling and targeting these pathways can be of chemotherapeutic benefit.

Aberrant and deregulated metabolism

Warburg effect

Dr. Otto Heinrich Warburg is credited with discovering the phenomenon that cancer cells switch to a glycolytically active environment with lactic acid fermentation occurring within the cytosol (485). This is the opposite to what is observed in normal cells as normal cells have low rates of glycolysis and rely on oxidative phosphorylation in the mitochondria for energy (485). He also postulated that this change is not a passive consequence of tumorigenesis, but a potent tumorigenic driver and this is called the Warburg hypothesis (Figure 32) (485). Cancer cell metabolism is indisputably due to aberrant regulation and expression of tumor suppressor genes and oncogenes. Glycolysis and oxidative phosphorylation are two vastly different forms of producing energy with differing cellular locations, enzymes, energy yielding rates, and kinetics (485,486). Glycolysis is a very rapid, normally anaerobic, cytosolic, method of producing instant energy and is a very evolutionarily ancient pathway, being conserved from humans to the most primitive prokaryotic species (485,486). This process is very inefficient and yields only two ATP molecules per molecule of glucose.

Oxidative phosphorylation produces 32-33 molecules of ATP per glucose, is aerobic, takes place in the mitochondrial matrix/mitochondrial intermembrane, and is not as expedient as glycolysis (487). Depending on the cell type, many cells are very energetically demanding (i.e. muscle, pancreatic, brain) and require

large amounts of energy in the form of adenosine triphosphate (ATP), which is the major energy source of most organisms, including human cells. ATP is an excellent energy source, which is released via ATP hydrolysis, to either ADP or AMP.

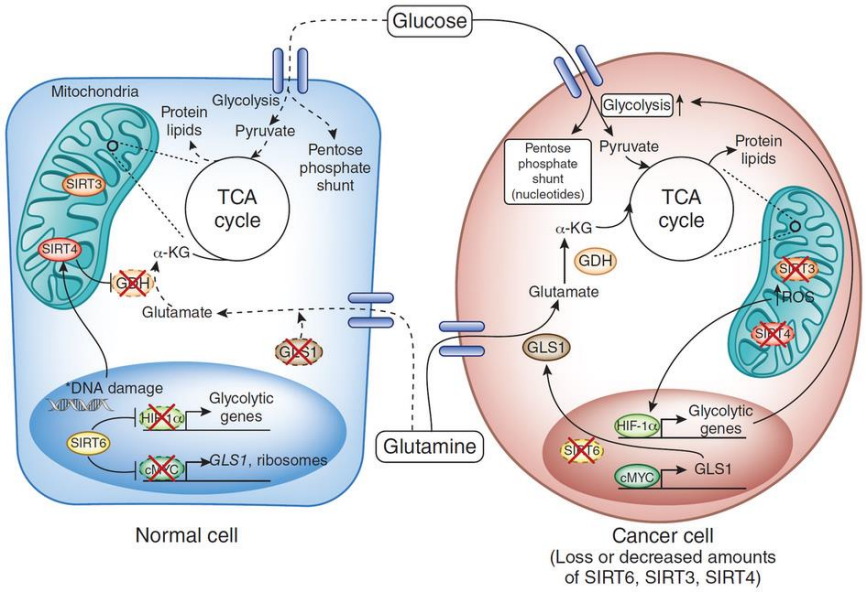

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Figure 32: Warburg effect. The Warburg effect is a metabolic shift that occurs within cancer cells. In normal cells, the majority of energy is derivd by oxidative phosphorylation and very little glycolysis. This is also emphasized by the low expression of HIF-1α and c-Myc, which upregulate glycolytic genes. Cancer cells undergo the Warburg effect and upregulate glycolytic genes by overexpression of HIF-1α and c-Myc and decreased oxidative phosphorylation. There is also increased glucose influx by upregulation of GLUT Transporters, increased pentose phosphate pathway metabolism, and upregulation of glutaminases. These cancer cells also lose expression of sirtuins SIRT6 SIRT3 and SIRT 4.

The ATP/AMP ratio is a metabolic indicator and is directly proportional to the energy state of the cell (486,487). When ATP/AMP ratio is high, anabolic pathways are activated, such as the mTOR pathway, PI3K/AKT, FAS (fatty acid synthase), protein synthesis through ribosomal biogenesis, and other pathways (487,488). AMP kinase (AMPK) is a mediator of a low energy state and inhibits

anabolic pathways regulated by mTOR. AMPK can be activated by AMP allosterically activating AMPK by binding to its CBS domain within the γ-subunit, although phosphorylation by LKB1 (a component of the heterotrimer AMPK kinase) on T172. When normal cells become transformed, they exhibit the characteristic of switching from aerobic oxidative phosphorylation to anaerobic/aerobic glycolysis (154,155,487). This involves shunting a key metabolite that is critical to oxidative phosphorylation and the TCA cycle, namely pyruvate.

Pyruvate dehydrogenase complex

Pyruvate is the end byproduct of glycolysis and the result of the transamination reaction of alanine. In normal cells the pyruvate dehydrogenase complex, includes: pyruvate dehydrogenase (E1), Dihydrolipoyl transacetylase (E2) and dihydrolioyl dehydrogenase (Figure 33). E1 has a dual role by decarboxylating pyruvate and acetylating lipoic acid (which is covalently bound to E2) to lipoamide (489). Like the majority of enzyme that decarboxylate substrates, E1 utilizes thiamine pyrophosphate (TPP) as a cofactor (489). TPP is unique in the fact that within the thiazolium ring there is a resonance structure that generates a carbonium anion ylide, which is very reactive and nucleophilically attacks (S_N2) the carboxylate of pyruvate.

PDH is very critical in the metabolism of pyruvate and shunting it into aerobic mitochondrially driven pathways, and its activity is regulated by phosphorylation and dephosphorylation, pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase

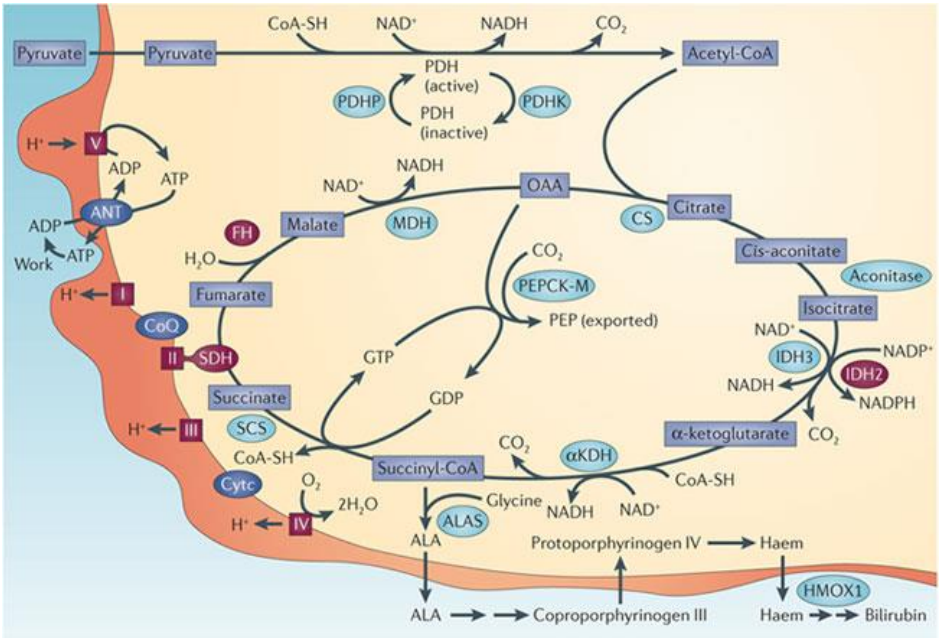

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Figure 33: Pyruvate dehydronase complex. This complex consists of E1: pyruvate dehydrogenase, E2: Dihydrolipoyl transacetylase, and E3: dihydrolipoyl dehydrogenase. It is negatively regulated by phosphorylation by pyruvate dehrydrogenase kiase (PDK) and positively regulated by pyruvate dehydrogenase phosphatase 1 (PDHP1). Phosphorylation of PDH is inhibitory and is stimulated allosterically by NADH, citrate and acetyl-CoA, and allosterically repressed by pyruvate, CoA, and NAD+. PDP1 is inhibited by magnesium and calcium ions. PDK1 is a drug target in cancer and can be targeted by use of chemicals such as dichloroacetic acid (DCA). In cancer mitochondrial DNA mutations are often reported for Coomplex 1-V of oxidative phosphorylation and pyruvate dehydrogenase.

phosphatase (PDP1) (489). Phosphorylation of PDH is inhibitory and is stimulated allosterically by NADH, citrate and acetyl-CoA, and allosterically repressed by pyruvate, CoA, and NAD+. PDP1 is inhibited by magnesium and calcium ions

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Pyruvate/Lactate ratio and oxidative phosphorylation vs. aerobic glycolysis

Cancer cells shunt pyruvate from aerobic metabolism by increasing the expression of lactate dehydrogenase (489). This enzyme is responsible for the reversible interconversion of pyruvate to lactate. With increases glycolytic flux, there is increased production of NADH, which pushes the equilibrium of pyruvate/lactate to the production of lactate, which is shuttled out of cancer cells by the protein symporter MCT4 (monocarboxylate transporter 4) (492). This efflux of lactate is accompanied by efflux of H+, making the cell less acidic and preventing ketoacidosis in the cell. Another protein transporter that is upregulated in cancer cells is the NA+/H exporter 1 (NHE1) (493). This is an antiporter that cooperates with MCT4 by increasing the efflux of H+ by increasing the influx of Na+, which is energetically favorable and goes with the Na+ concentration gradient (492). In normal cells after pyruvate is shuttled to the mitochondria and acted upon by TCA cycle enzymes, generating 3 NADH molecules, a GTP (guanosine triphosphate) and a QH2 (the reduced form of ubiquinone, ubiquinol) (489). NADH and ubiquinol participate in the electron transport chain (ETC) which generates a proton gradient used by ATP synthase to generate 32-36 ATP molecules per glucose molecule.

These enzymes are present in the mitochondrial inner membrane on the side facing the mitochondrial matrix and include complex I (NADH:Ubiquinone oxidoreductase), complex II (succinate dehydrogenase, which also participates in the TCA cycle), complex III (cytochrome c oxidoreductase), and complex IV (cytochrome c oxidase) (489,490). These enzymes transfer electrons from NADH to ubiquinone, to FADH and then finally to oxygen, generating water as a final byproduct. NADH as stated is generated from the TCA cycle as a result from the TCA cycle (489,490). The acetyl-CoA generated by the PDH complex is used by citrate synthase to generate oxaloacetate, which is the start and finish of the cycle (489,490). This metabolic hub ensures complete oxidation of pyruvate and requires only a catalytic amount of oxaloacetate, as radiolabeling has demonstrated that the two carbons given by acetyl-CoA are completely oxidized by this cycle into carbon dioxide. However, cancer cells switch from oxidative phosphorylation to glycolysis, which is used in anaerobic, hypoxic/anoxic environments (489,490). This has long been a paradox in cancer biology however there are some observations that can explain this switch.

Along with providing ATP, glucose provides constituents for other metabolic pathways. Glucose can also undergo the pentose phosphate pathway (PPP), which is utilized to generate ribose 5 phosphate for nucleotide synthesis (ribose-5-pyrophosphate in the purine *de novo* synthesis pathways) and the anabolic counterpart to NADH, NADPH (489,490). NAPDH participates in fatty acid synthesis as well as steroidogenic pathways. NADPH also participates in

GSH-mediated antioxidant pathways and therefore increased glucose uptake increases the concentration of GSH and the cancer cells ability to neutralize deleterious ROS and this makes the cancer cell more stable in hypoxic environments (494).

TLK1/pentose phosphate pathway and GLUT transporters/glycolysis

The pentose phosphate pathway has two stages, the oxidizing stage (which includes glucose 6-phosphate dehydrogenase) and the non-oxidative phase, which involves transaldolases and transketolases (495). Transaldolase combines sedoheptulose 7-phosphate with glyceraldehyde-3-phosphate to generate fructose-6-phosphate and erythrose-4-phosphate (495). Transketolases participate in the first and last step on the non-oxidative phase of the PPP, generating sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate from ribose-5-phosphate and xylulose-5-phsphate in the first step and then glyceraldehyde-3-phosphate and fructose-6-phosphate in the third step (495). These resulting molecules are substrates for glycolysis and serve as anaplerotic flux and the enzyme transketolase 1 (TLK1) is a cancer biomarker and a therapeutic target (Figure 33) (496).

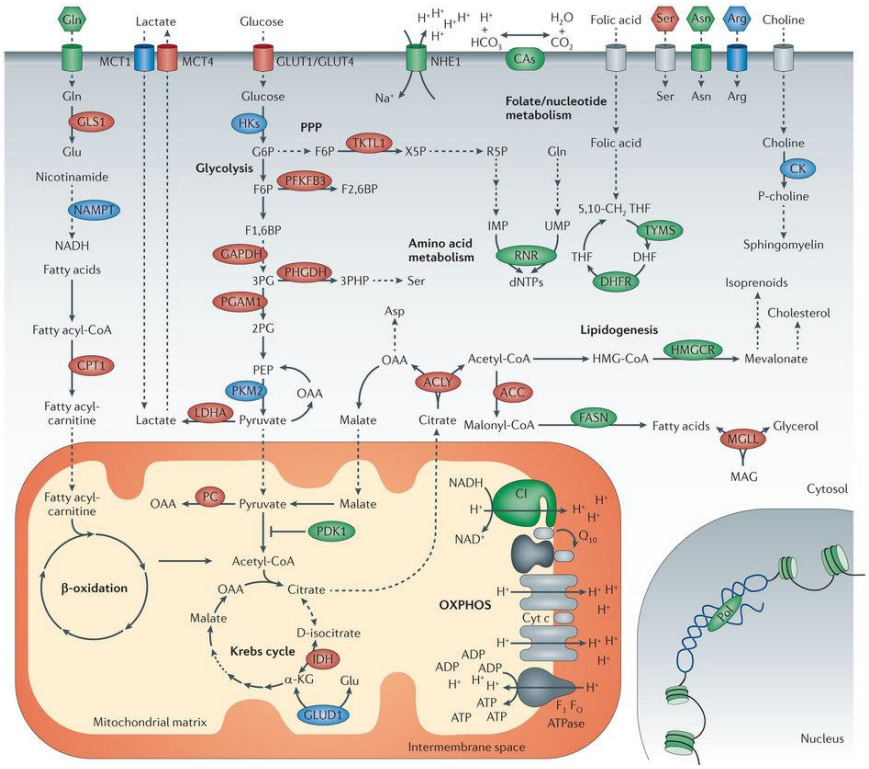

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Figure 34: Transketolases and the pentose phosphate pathway. There is an increase in the pentose phosphate pathway (PPP) in cancer cells and this involves upregulation of transketolase 1 (TLK1), which is nvolved in the non-oxidative phase of PPP. Transketolases participate in the first and last step on the non-oxidative phase of the PPP, generating sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate from ribose-5-phosphate and xylulose-5-phsphate in the first step and then glyceraldehyd3-3-phosphate and fructose-6-phosphate in the third step. These resulting molecules are substrates for glycolysis and serve as anaplerotic flux Glucose uptake is also increased by upregulation of GLUT 1,3, and 4, which is upregulated by c-Myc and HIF-1α. HIF-1α also serves as a transcription factor that upregulates many glycolytic enzymes (including hexokinase 1 and 2 near VDACs situated at the mitochondrial outer membrane), phosphofructokinase 1 and 2, and pyruvate kinase, which are key "commitment steps" of glycolysis. Compounds such as 2-DG (deoxyglucose), which inhibits hexokinase and 3-BP (3-bromopyruvate), which potently inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a vital enzyme in glycolysis that generates ATP using NADH. 3-BP is currently in Phase 1 clinical trials for liver cancer and should show promise in other metabolically overactive cancers such as pancreatic cancer and RMS (rhabdomyosarcoma). HIF1-α also upregulates PDK1, which dismantles pyruvate from entering the TCA cycle by virtue of its ability to inhibit PDH, LDHA, MCT4, NHE1, and carbonic anhydrases 9 and 12, which couples protons exported by NHE1 and MCT4 to bicarbonate, generating carbonic acid which breaks down into water and CO_2. These genes have consensus sequences within their promoters that contain HIF-1α and c-Myc binding motifs.

Ectopic expression of TLK1 induces the Warburg effect in melanoma cells and enhances malignancy by DNA-hypomethylation (497). Cancer cells also

handle the energy demand predicament by upregulating glucose transporter proteins, namely GLUT1, GLUT2, GLUT3, and GLUT4 (Figure 34) and this is a major distinguishing feature between transformed and normal (498,499). Drugs such as WZB117, ribonavir, phloretin, and STF-31 inhibit these transporters (500-504). With an increase in the influx of glucose, there is an accompanying upregulation of enzymes involved in glycolysis. Cancer cells become very hypoxic and this increases expression is HIF-1α and both HIF-1α and c-Myc (326,327) serve as transcription factors that upregulate many glycolytic enzymes (including hexokinase 1 and 2 near VDACs situated at the mitochondrial outer membrane), phosphofructokinase 1 and 2, and pyruvate kinase (505).

Aldolase [which cleaves fructose 2, 6 bisphosphate into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP)], triose phosphate isomerase and phosphoglycerate mutase are also upregulated, but these enzymes also participate in gluconeogenesis (489, 490). Compounds such as 2-DG (deoxyglucose), which inhibits hexokinase and 3-BP (3-bromopyruvate), which potently inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a vital enzyme in glycolysis that generates ATP using NADH are drugs that have and are currently being investigated as anticancer agents (506,507). 3-BP is currently in Phase 1 clinical trials for treating liver cancer and should show promise in other metabolically overactive cancers such as pancreatic cancer and RMS (rhabdomyosarcoma) (507). HIF1-α also upregulates PDK1, LDHA, MCT4, NHE1, and carbonic anhydrases 9 and 12 (326,327). These genes have

consensus sequences within their promoters that contain HIF-1α and c-Myc binding motifs (326,327, 508).

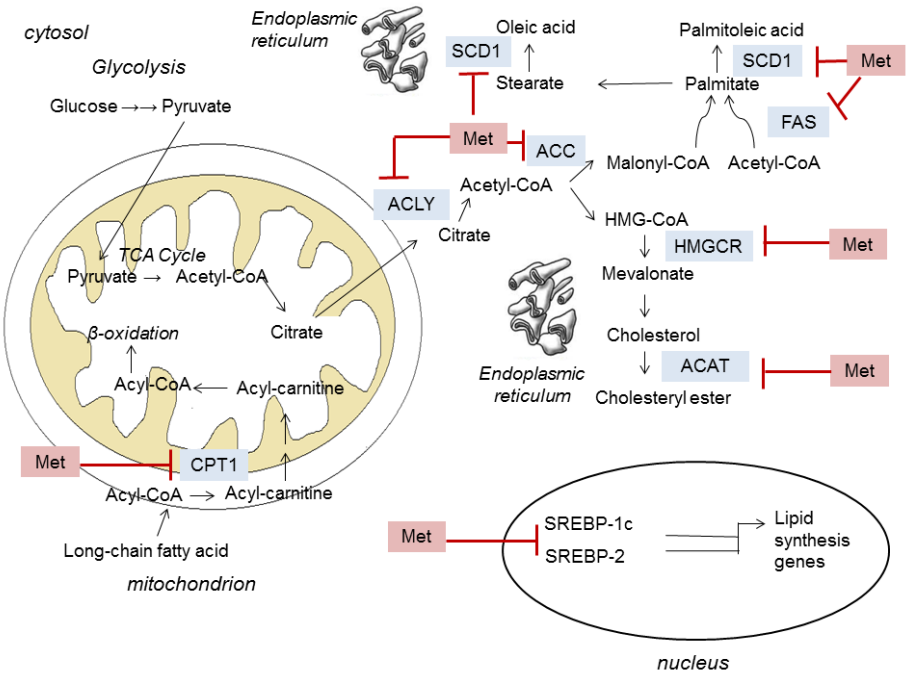
In cancer cells, there is also decreased expression and function of glycolytic repressors. Along with regulating DNA damage responses, apoptosis, and the cell cycle, p53 also has a substantial role in metabolism and suppresses the uptake and metabolism of glucose. The GLUT1 and GLUT 4 genes are directly repressed by p53 and GLUT 4 is indirectly inhibited by p53's negative regulation of NFκB (188,509,510). P53 also represses expression of PGM and induces expression of SCO2 (SCO2 cytochrome c oxidase assembly member), TIGAR (TP53 induced glycolytic and apoptotic regulator), and GLS2 (glutaminase 2) (509,510). SCO2 and GLS2 potently enhance oxidative phosphorylation pathways, with the former being directly involved in the assembly and stimulation of Complex IV in the ETC. TIGAR is the negative counterpart of the enzyme PFK2, which converts fructose-1-phosphate to fructose 2,6-bisphosphate (509). Fructose 2,6 bisphosphate potently allosterically activates PFK1, therefore TIGAR serves as a potent negative regulator of PFK1, which is an enzyme involved in an indispensable step in glycolysis (509,510). All these enzymes are repressed in cancer cells, some in part due to loss of p53 activity.

Glutaminases

In addition to increased glycolytic flux in cancer cells, there is also increased protein translation, due to enhanced mTOR activity, and increased

glutaminase activity, which converts glutamine into glutamate, and then which is metabolized to glutarate. Glutarate is the ketogenic counterpart of aspartate, and can be shuttled into the mitochondria as α-keto glutarate (511). In cancer cells there is increased expression of glutaminase and drugs such as BPTES (Bis-2-(5-phenylacetoamido-1,3,4-thiadiazol-2-yl)ethyl sulfide and CB-839 are potent glutaminase inhibitors that are in Phase 1 clinical trials (512). Inhibition of glutaminases (i.e. by BPTES) is effective in treating many solid cancers, such as glioblastoma and CB-839 has also shown promising results in TNBC (triple negative breast cancer) (512). CB-839 is also in Phase 1 clinical trials for other solid tumors such as NSCLC (513). Derivatives of CB-839 (i.e. Pac-CB, CBE, CB-Erl, and CBD) are in Phase 1 trials for NSCLC, renal cell carcinoma (RCC) mesothelioma, fumarate-hydratase (FH) and/or succinate dehydrogenase (SDH) tumors of the gastrointestinal tract and stroma, and tumors haring c-Myc amplifications or mutations in isocitrate dehydrogenase (IDH1 and IDH2) (514-516). Drugs are also used to target ASC amino acid transporter 2 and LATS1, which import glutamine into the cancer cells (517). Drugs such as α-MT and SERMs inhibit these channels (518).

ATP citrate lyase, and carnitine palmitoyl transferase 1a (CPT1a)



Cantoria MJ, Patel H., Boros LG , Meuille EJt, *http://www.intechopen.com/books/pancreatic-cancer-insights-into-molecular-mechanisms-and-novel-approaches-to-early-detection-and-treatment/metformin-and-pancreatic-cancer-metabolism.* 2014 (520)

Figure 35: ATP citrate lyase, SREBP1, and CPT1a. Cancer cells increase mTOR activity and have an increase in amino acid metabolism. This is accompanied by not only the activatation of Rag A/B and C/D but the overexpression of glutaminases. In normal cells glutaminases convert glutamine to -keto-glutarate and then glutarate, which is shunted to the malate/asparte shuttle within the mitochondria. However in cancer cells this pathway is attenuated and the resulting glutarates are used for amino acid synthesis. There is also an upregulation of faty acid synthase (FAS), ATP citrate lyase and a concomitant decrease in carnitine palmitoyl transferase 1 (CPT1) and uncoupling proteins (UCP) such as UCP2 and UCP3. Along with increase lip synthesis there is also an increase in the anepleurotic flux into the mevalonate pathway. This is important for cancer cells for membrane synthesis (cholesterol) and prenylation (farnesylation and geranylgeranylation) of oncogene like ras. Drugs like metformin inhibit these pathways and have potential as anticancer agents.

Fatty acid synthesis and lipogenesis are critical pathways upregulated in cancer, with increased cell cycle activity and mitosis there is an increased need for membrane synthesis and cancer cells express high levels of the enzyme ATP citrate lyase and fatty acid synthase (FAS) (519). There is also a concomitant

decrease in the enzyme carnitine palmitoyl transferase 1A (CPT1A), a transporter protein that transports fatty acids into the mitochondria (Figure 35) (520). Carnitine serves as a shuttling intermediate for MCTs (medium chain triglycerides) and other fatty acids (520). The mitochondria are the location where fatty acids are oxidized to yield energy by the process of β-oxidation. In many tumors, the expression of β-oxidation enzymes is decreased as well as the expression of CPT1 (520). However, there is evidence that the role of CPT1 in carcinogenesis is necessary and therefore the absolute role of this protein and fatty acid oxidation is equivocal. Cancer cells have a high-energy demand for ATP, and ABC transport proteins such as MDR1, are important in cancer and confer chemotherapeutic resistance (521). ATP production inhibits anoikis, which is cellular death induced by lack of cellular attachment to the ECM or some other substratum. Mitochondria require CPT1 for fatty acid oxidation and this protein also confers mTOR inhibitor resistance and suppresses Bax/Bak induced apoptosis (520). Increased β-oxidation can increase reactive oxygen species production, and is inhibited by drugs such as metformin (Figure 35). Tumors with increased CPT1 production there is also accompanied increased expression of uncoupling proteins (UPC2, UPC3), which dissipate the proton gradient generated by ETC as heat (Figure 35) and drugs such as dinitrophenol have similar effects and are very mitotoxic (522,523). These experimental findings suggest that the role of CPT1 in carcinogenesis is cancer and tumor cell specific (520,524).

Fatty acid synthase (FAS)

FAS along with sterol response element binding protein-1 (SREBP-1) are involved in fatty acid synthesis, with SREBP-1 being a major transcription factor for genes involved in lipid and sterol synthesis that possess a sterol response element (SRE) (525). FAS is not a single enzyme but a complex of enzymes that like β-oxidation perform stepwise reaction involving the addition rather than the lysis of two carbon atoms (520,521). This reaction uses acetyl-CoA along with malonyl-CoA to generate the 16-carbon fatty acid palmitate. Palmitate is involved in the post translation modification of many proteins, including sonic hedgehog (Shh), which is N-palmitoylated and is a potent oncogenic factor in breast cancer, glioblastoma, and other cancers (526). There are two isoforms of FAS, FASI and FASII, with the former being the eukaryotic form and the latter being the prokaryotic form (520). The first two reactions catalyzed by FAS involve the priming of acetyl CoA and malonyl CoA to the protein acyl carrier protein (ACP), which is also upregulated in tumor cells. Other FAS enzymes then form the product butyrl-COA by catalyzing reactions of these primed CoA intermediates. This is the beginning product of FAS, which is subsequently elongated to form palmitate (palmitic acid). Palmitate and related fatty acid stearate (18-carbon fatty acid) also accumulate in cancer and can lead to fatty acid disease or steatosis of the liver, which can increases the likelihood of HCC. SREBP1-c also regulated the transcription of ACC, and the active form SREBP1c is upregulated in cancer and leads to hyperactivation of ACC as well as FAS (520,521,525).

Mevalonate pathway: SREBP and HMGCoa reductase

SREBP1 and SREBP2 are helix-loop-helix (HLH) transcription factor that remains inactive on the endoplasmic reticulum and require cleavage for activation (525,526). AMPK, p53, and Rb negatively regulate SREBP in a normal cell (525,526). The enzyme A citrate lyase (ACP) which is upregulated in cancer can cleave citrate to generate acetyl CoA, which feeds into the mevalonate pathway (Figure 35) (527). Mevalonate is a critical intermediate for synthesis of cholesterol, which is an important membrane constituent that influences membrane fluidity of the membrane and this has a direct effect on lipid raft formation and activation of Ras and AKT (527,528). Drugs called statins, which treat hypercholesterolemia by inhibiting the enzyme HMG-CoA reductase (HMGCR), target the mevalonate pathway that is involved in cholesterol synthesis and consequently exhibit antitumorigenic activity (527,528). This enzyme catalyzes the reaction of HMG-CoA to mevalonate and is critical for all steroid synthesis. Mevalonate is an aneuplorotic intermediate not only for steroid synthesis but also for prenylation-dependent targeting of proteins (527,528). A biotin-dependent decarboxylase (as with many decarboxylases) generates the isopentyl pyrophosphate (IPP) intermediate that is in equilibrium with dimethylallyl pyrophosphate (DMAPP) by IPP isomerase activity, which are used for formation of farnesyl and geranyl groups that serve as tethers for proteins such as Ras. The enzyme farnesyl diphosphate synthase (FPS) generates geranyl-PP then

farnesyl-PP sequentially, which is the key intermediate to cholesterol, ubiquinones, sterols, dolichols, and prenylated proteins (527,528). The formation of squalene is required for all steroids, including cholesterol, which is itself an intermediate to all steroid synthesis and is formed into a pregnane (pregnenolone) by the cholesterol debranching enzymes (527,528). This can lead to the formation of estradiol and testosterone (or dihydroxy testosterone formed by 5α-reductase), which can be deleterious and enhance breast cancer and androgen receptor positive prostate cancer respectively. Farnesyl-PP and prenylation play a critical role in cancer as well (529).

Farnesyl transferases as cancer drug targets

Farnesylation of Ras is critical for its full activation and geranylgeranylation of Rac, Rho, cdc42 (monomeric GTPases involved in actin cytoskeletal stiffening and dynamics), Rab (vesicular trafficking proteins), and Rap (530). These prenylation reactions are promoted by farnesyl transferases (FT) and geranylgeranyl transferase enzymes. Farnesyl transferase adds a 15-carbon sesquiterpenoid group to Ras and other proteins bearing a CAAX motif within their carboxy terminus (530). Ras has been deemed “undruggable” as many drugs that target Ras kinase activity are inefficacious or become obsolete due to tumor cell resistance. However, FT inhibitors have garnered much attention and many are being used in research and clinical trials. For example the drug tipifarnb, is being used or elderly patients with AML (531). The drug lonafarnib is being used to treat breast cancer in conjunction with anastrazole and tamoxifen and their

efficacy in treating colorectal and pancreatic cancers is also being investigated (532). These drugs are also being studied in thyroid, bladder, and kidney cancers as they also target H-ras, which is the driver of those cancers (533-536). With the success of FT-inhibitors there also comes resistance to these drugs. Disappointingly, in many cases mutated Ras (both K- and H-) can become geranylgeranylated (530,537). Many leukemias and also colon, pancreatic, kidney, thyroid, melanoma, and bladder cancers are also affected substantially by geranylgeranyl transferase (GGT) -inhibitors (538-543). Many GGT inhibitors including GGTI-297, GGTI-298, and VHEMBL525185 are being investigated in multiple tumor types (538-543). Recently it was shown that the turmeric anticancer compound curcumin, was able to bind with high affinity to both FT and GGT1 suggesting a potential anti-Ras activity for curcumin (544). Statins inhibit not only geranylgeranylation by depleting levels of GGP, but also induce expression of p27Kip1 and p21Cip1 by inducing the degradation of Skp2, which is a subunit of the SCFSkp2 ubiquitin ligase that targets p27Kip1 and p21Cip1 for proteasome-dependent degradation (545,546). FT-inhibitors and GGT1 inhibitors also have effects on Hippo signaling by negative regulation of Hippo signaling antagonists YAP and TAZ, which are overexpressed in some cancers, and exhibit antitumorigenic activity in Hippo mutated cancers (547).

Breast Cancer

Breast cancer classification

Breast cancer is any cancer that originates and develops in the mammary glands and breast tissue (9,10). It is the most common cancer in women and the second most common cancer worldwide after lung cancer (20,21). The global burden of breast cancer is greater than all other cancers and incidence rates are increasing (20,21). The beast tissue is made of adipose and connective tissue, lymph nodes and blood vessels. Breasts have a series of glands called lobules that produce milk and thin tubes called ducts that carry the milk from the lobules to the nipples where they can feed young (20,21). The most come types of cancer are ductal carcinomas, which can be broken down into the categories of non-invasive and invasive cancers. Non-invasive breast cancer consists of ductal carcinoma in situ (DCIS), which is a tumor that has grown to the boundaries of the ECM, has not become invasive, and originates in the milk ducts (548). Invasive ductal carcinomas are those in which the cancer cells have invaded the basement membrane and can metastasize to distal tissues (548). This kind of cancer is observed in about 80% of breast cancer patients and involves cancers that have originated in the milk ducts. These can be further broken down into five subcategories: Tubular carcinoma, medullary carcinoma, mucinous carcinoma, papillary carcinoma, and cribriform carcinoma. Tubular carcinoma originates in the milk duct and spreads into healthy tissue; however, these tumors are usually low grade and are somewhat differentiated “normal” looking cells that grow very

slowly (548,549). Medullary carcinoma is a very rare cancer that has superficial similarity to the medulla of the brain hence the name. It effects women in their late 40-early 50s and more common in women that have a BRCA1 mutation (550). Although they appear aggressive, they grow slowly and have minimal invasive behavior. Mucinous, or colloid, carcinomas are cells that are rich in the protein called mucin. Mucins are proteins that are involved in carcinogenesis and are upregulated in advanced carcinomas (551). This type of cancer is common in women who have gone through menopause and responds well to treatment (551). Papillary carcinomas affect 1-2% of invasive breast cancer patients and it is characterized by the presence of well-defined finger like projection that are moderate in tumor grade (552). Cribriform carcinoma involves cancer cells that invade the stroma in nest like formations and have conspicuous holes within the tumor (553). With both papillary and cribriform invasive ductal carcinomas, DCIS is also present concomitantly (548,549,552,553).

Along with invasive ductal carcinoma, there is also invasive lobular carcinoma, which is the second most common type of breast cancer (~10% of breast cancer patients) (554). This type of cancer originates in the breast lobules and has a 5-year survival rate of 85%. These kind of cancer has multiple histological patterns, which include classical, alveolar, solid, tubulolobular, pleomorphic, and mixed (554). The two most common patterns are classical and mixed. Classical is composed of round cells that have very large nuclei and very little cytoplasm, having a single file infiltrating pattern (554,555). Mixed simply

implies that the cells represent multiple histological patterns and/or ones that have no distinct or dominant pattern. Pleomorphic pattern is like classical but the cells are pleomorphic (not uniform) in cell size, morphology, and cytoplasmic to nucleus ratios (554,555). The next common type represents 10% of ILC and these are solid tumor cells, which are similar to classical cells except they are arranged in sheets that are intercalated by sheets of intervening stroma (554,555). The least common histological patterns are alveolar and tubulolobular presentations the alveolar tumors form as aggregates of classical-like cells and have morphologies that are similar to alveolar cells of the lung (556).

Breast cancer staging

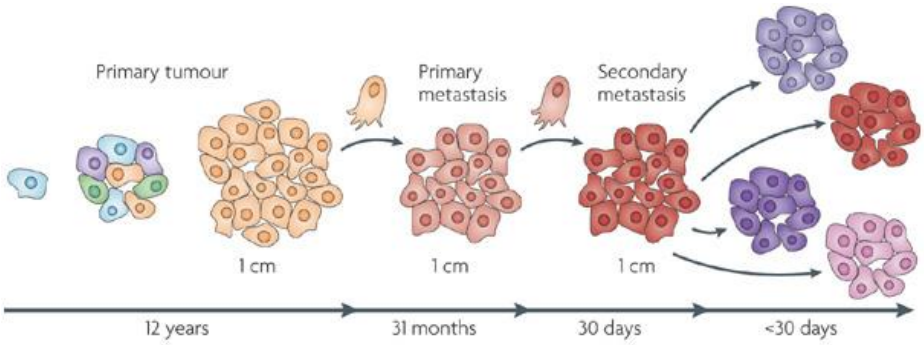


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Figure 36: Breast cancer staging. Stages for breast cancer malignancy.

Breast cancer classification uses a staging system what is known as the TNM system (**T**umor, Lymph **N**odes, **M**etastases) (557). This classification system takes into account the size of the tumor, its boundaries and whether it has infiltrated stroma and other components of the ECM, if it has invaded the lymph

nodes, and if there are any metastases (Figure 36). Tumor stages can be classified T0-T4 (557). TO means that the tumor cannot be assessed for staging and the classification Tis is carcinoma in situ (either ductal or lobular). There is also a special kind of Tis for Paget's disease, which is associated with the nipple and the surrounding parenchyma. T1 can be broken down into T1a, T1b, T1c, and Tic, and all of these subdivisions are contingent on the size of the tumor, with T1a: 1-5 cm, T1b: 5-10cm, T1c 10-20 cm (557). T2 and T3 also are based on tumor sizes with 20-50 cm being T2 and sizes greater than 50cm belonging in the T3 category (557). T4 is a category that is devised for tumors that have begun to invade into the chest wall through skin and muscle. T4a there is extension into the pectoralis muscles and T4b tumors exhibit ulceration and edema (peau d'orange) with ipsilateral site nodules (557). T4c is a combination of T4a and T4b and T4d is inflammatory carcinoma (557).

The development of breast cancer involves a progression through a series of intermediate processes, which start with ductal or lobular cell proliferation, followed by carcinoma in situ, invasive carcinoma, and then metastatic cancer, which can metastasize preferentially to the lungs and bones (51-54). Depending on the type and cancer and its stage there are varying degrees of survival, prognosis, and chemotherapeutic response and there are several diagnostic markers for breast cancer. Diet, exercise, and family history also play a significant role in breast cancer onset (558). Breast Cancer was originally called "Nun's disease" which was linked to abstinence from sex, a risk factor for breast cancer.

One of the most prominent markers of breast cancer is the estrogen receptor (ERα), which represents about 60% of breast cancer patients (559). These cancers have good prognosis and can be treated with drugs such as tamoxifen and clomifene (559). Cytotoxic drugs such as 5-FU (5-fluorouracil) and Adriamycin (doxorubicin) are also used to treat breast cancer (560). In certain cases patients undergo a unilateral or bilateral mastectomy to remove the tumor (20,21). ER negative breast cancers are much more difficult to treat and are refractory to chemotherapy (559). The heterogeneity of breast cancer is emphasized in comparative genomic hybridization data demonstrating clear and somewhat ambiguous associations (559,561). A more comprehensive approach integrating genomics, proteomics and potentially transcriptomics will lead to the innovation of better chemotherapeutic strategies that are more specialized to a specific cancer subtype (561). Breast cancer cell lines (as well as cancers) can be broken down into five basic categories based on their molecular infrastructures: Luminal A and B, Basal, Claudin-low, and HER-2 overexpressing. Luminal A and B cancer types are usually very response to chemotherapy and have good prognosis in a clinical setting (562). The expression of Ki67 (a proliferative marker) is variable as well as the expression of HER2. They express both ER and PR. Basal and claudin low are triple negative cell lines/tumors that are not endocrine response with variable chemotherapeutic responsiveness (562). In the following sections, known biomarkers of cancer will be discussed as well as how they are targeted.

Nuclear receptors and breast cancer: estrogen receptor (ER) and progesterone receptor (PR)

More than 700,000 of breast cancer patients are positive for elevated or aberrant expression of hormone receptors (20,21,558,563). Hormone receptors are receptors, which dimerize (either in a hetero- or homo-dimeric fashion) and bind to their respect response elements activating expression of hormone response genes (563). Steroid hormone receptors are expressed in both the epithelium and the breast stroma and breast tumors are initially characterized by expression of ERα (558,563). The PR (progesterone receptor) also plays a prominent role in breast cancer and both PR and ER can be tested for expression by immunohistochemistry analysis and their expression has important clinical and treatment implications (563,564).

There two different estrogen receptors: ERα and ERβ (Figure 40). The hormone estradiol (E2) binds with equal affinity to both ERα and ERβ. There are other estrogenic steroid hormones, namely estrone and estriol, with affinities for ERα and ERβ respectively (558,564,565). There are also drugs that can target these different ER isoforms and these are called selective estrogen receptor modulators (SERMs) and SERMs can be ER agonists or antagonists and their ER activity is dependent on tissue (558,565). For example, the compound tamoxifen is an ER antagonist in breast cancer while it is an agonist in other tissues (558,565). Another drug used to treat advanced stage metastatic cancer is the full ER antagonist fulvestrant. Certain compounds such as genistein found in soy

bind preferentially bind to ERβ and exhibit tumor suppressive roles in colon cancer (558,565). ERα and ERβ are encoded on two different chromosomes and have unique tissue distribution, with the alpha isoform predominantly found in breast, endometrium, ovarian stroma, hypothalamus, and the efferent ducts (males) and the beta isoform being expressed in the brain kidney, granulosa cells, bone, heart, prostate, endothelium, and colon (566-573).

Like other nuclear receptors, ER remain inactive in the cytosol and upon binding to ligand translocate as hormone dimers to the nucleus and bind to inverted repeats that compose estrogen response elements (558,564,565). ER isoforms can are also involved in ligand independent regulation, which involves phosphorylation of its modular structure (558,564,565). For example, Akt phosphorylates S255 in DNA binding domain of the beta isoform and cdk2 and ERK phosphorylate S106 and S118 respectively in AF1 domain of ERα, which expedites the cell cycle (574). In breast cancers where ER expression is elevated the use of anti-estrogens (such as tamoxifen) and anti-aromatases (anastrazole) show the most chemotherapeutic benefit (558,565). Tamoxifen itself is a prodrug, which is metabolized by the action of cytochromeP450 enzymes CYP2D6 and CYP3A4 into 4-OH tamoxifen (afimoxifene) and N-desmethyl-4-OH tamoxifen (endoxifene) (558,559). These metabolites bind to the receptor and compete with estradiol. These metabolites can also recruit corepressor proteins such as NCoR (nuclear receptor corepressor 1) and SMRT (silencing mediator for retinoid or thyroid hormone receptors), which is also known as NCoR2, which recruit histone

deacetylases (575). However, upregulation of other receptors such as HER2/ErbB2, are associated with tamoxifen-resistant breast cancers and the upregulation of these growth factors must be suppressed and accounted for (576). In order for tamoxifen to suppress growth factor upregulation it requires the activity of PAX2 (paired box 2) transcription factor, which can promote a complex of tamoxifen bound ER with PAX2. However, the level of PAX2 must surpass another protein, AIB1 (amplified in breast 1, also known as NCOA3, TRAM-1), which can actually transform tamoxifen bound ER into a transcriptional activator of EbB2, by recruiting histone acetyl transferases to the ErbB2 promoter (577). This illustrates the degree of sophistication and involvement the intracellular milieu has in certain chemotherapeutic drugs and their effects on breast cancer targets. Anastrazole inhibits an enzyme known colloquially as aromatase (CYP19A1) (578). This is the only known enzyme in humans, as well as other mammals, to convert testosterone (as well as other androgens) into estradiol and other estrogens. Along with reversible inhibitors like anastrazole, there are also suicide inhibitors of aromatase such as exemestane (Aromasin), which forms a covalent bond in the active site of aromatase (578,579).

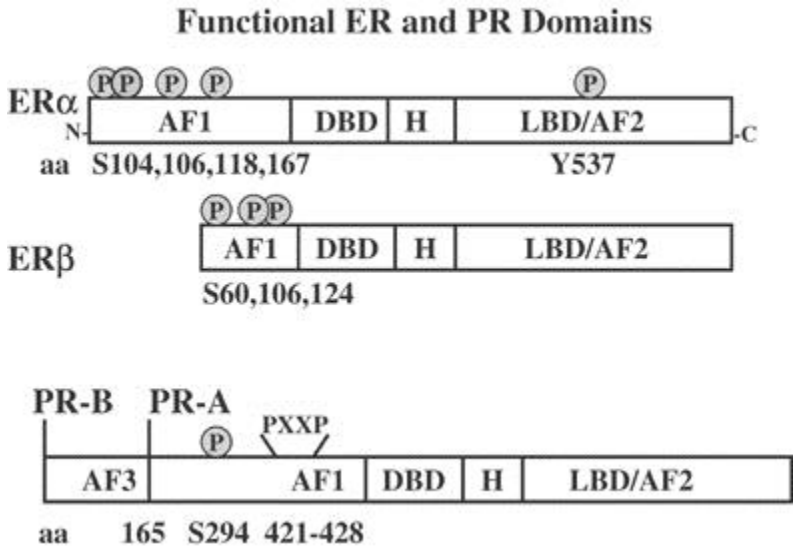

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Figure 37: SThe estrogen receptor and the progesterone receptor. structure and homology between the estrogen receptors (ERα and ERβ) and progesterone receptors (PR-A and PR-B)

Progesterone is an ovarian steroid hormone that is required for breast development, pregnancy, and lactation during pregnancy (564,580). This receptor is also has three different isoforms: hPR-A, hPR-B, and hPR-C and they are both dispersed in diverse tissues (Figure 37) (564,580). They are encoded from a single gene on chromosome 11 at q22-23, which has two different distal and proximal promoter regions (580). Using two internal translational start sites within a single mRNA encodes the full length protein (hPR-B), N-terminally truncated 9PR- A), and hPR-C (580). Most cells concomitantly express hPR-A

and hPR-B, which have different transcriptional effects with the same or distinct promoters (580). In the brain PR and progesterone control mating and reproductive behaviors, and development of the reproductive organs (580,581). PR-C lacks any transcriptional activity but can enhance activity of the other isoforms in a breast cancer context (580,582). Progesterone counterbalances the effects of estradiol, and progesterone like compounds (progestins) are frequently prescribed as contraceptives and for postmenopausal hormone replacement therapy to lower the risk of cancer associated with traditional hormone replacement therapy that had just estrogen (563,564,580-582).

The role of the PR in breast cancer is not completely understood either as a bonafide tumor suppressor or oncogene. The majority of breast cancer are positive for ER and PR (~60-70%) (558,563,564). These cancers can undergo autocrine-signaling mechanisms that can sustain cell proliferation. While many studies in breast cancer cell lines have established the role of PR and ER play in breast cancer cell proliferation, its physiological relevance in an organism (such as the human body) is ambiguous (558,559,563,564). Steroid hormone receptor mediated activation of cytoplasmic signaling molecules could theoretically serve to potentiate the nuclear function of these receptors. For example, amplification of PR nuclear functions may occur through rapid direct phosphorylation of PR proteins and/or their coregulators in repose to activation of PR-induced cytoplasmic pathways that coincide with ligand binding (563,564). Therefore appropriated phosphorylated and activated receptor complexes are orchestrated

for efficient and succinct regulation of selected genes, and this implicates a positive feedback loop for PR, which would explain the influence of activated signaling pathways on PR function (563,564).

Many pathways of PR are Src and MAPK dependent and upregulate expression of cell cycle dependent genes such as cyclins D1, E, and activate CDK2. Phosphorylation by both Src and MAPKs is required for PR to bind with Sp1 transcription factor to transactivate EGFR and p21 promoters (583,584). Activated oncogenes can also turn on PR pathways that are independent of ligand binding. Therefore, perhaps PR can promote breast cancer growth by synergistic steroid hormone action and activation of MAPK pathways. Similar mechanisms are seen with ligand activated ER which can induce a state of adaptive hypersensitivity, which is also an example of non-genomic signaling, where ER is membrane bound and not in the cytosol. In this model ligand-bound ER interacts with the adaptor protein Shc, phosphorylates it, which leads to recruitment of other adaptor proteins and induction of MAPK pathways such as the Ra/Raf/MEK/ERK (585). ERK then phosphorylates ETS factors and c-fos/c-jun (forming AP-1), which is independent of ER transcriptional activity (585,586). The PR can therefore use a similar mechanism in concert with MAPK pathways to enhance breast cancer tumorigenesis. Evidence to suggest this is in the fact that PR receptor expression is commonly associated with overexpression of cyclinD1, enhanced CDK2 activity and loss of tumor suppressor p21 (40%) (587). The PR is also known to directly interact with CDK2, cyclins A and E and its activity is

highest during S phase of the cell cycle and when p27 activity is lost (40% of cancers), PR activity becomes independent of ligand and is driven by CDK2 (587,588). Progestins are also known to induce cyclin D1 expression and induce re-entry of antiestrogen-induced quiescent into the cell cycle. Therefore, this suggests a role of PR in the cell cycle and its activity may become aberrant when cell cycle checkpoints are inactivated (563,585,587). Experiments where the *BRCA-1* gene was knocked out showed increased activity of PR and cells where p53 and BRCA-1 protein expression is lost the growth of tumors was attenuated by anti-progestins (587,588). These pieces of evidence definitely suggest a prominent role of PR in PR/ER positive breast cancer and further research must be done in order to fully elucidate mechanisms of PR in breast cancer.

ErbB2 and breast cancer

ErbB2 (also known as HER2, HER2/neu, EGFR2) is a member of the epidermal growth factor receptor family and is commonly amplified in breast cancers (51-51,562). HER2 is a transmembrane tyrosine kinase receptor that is encoded by the *ERBB2/HER2* gene on chromosome 17q21 and is amplified in 30% of breast cancers and is a negative prognostic factor for patient survival (51-54,562). HER2 lacks any endogenous ligands, however, when overamplified in cancer it can homodimerize ligand-independently or heterodimerize with ligand bound EGFR receptors, triggering autophosphorylation of the cytoplasmic tails and downstream RTK signaling (i.e. PI3K/AkT, MAPK pathways) (51-54,82,83,562). Crystallographic evidence shows that HER2 exists in a

constitutive ligand-bound confirmation, which can activate other receptors (563). There are currently drugs that target HER2 such as lapatinib and monoclonal antibodies such as trastuzumab (Herceptin) (563). Lapatinib is a kinase inhibitor, which inhibits the kinase domain of HER2 and EGFR and the trastuzumab antibody blocks receptor hetero-and homo-dimerization and may have other effects including attenuation of endocytosis of the receptor as well as cleavage of the extracellular domain, and activation of antibody-dependent cellular toxicity (ADCC) (563,564).

HER2 status is often assessed by IHC analysis or fluorescent in situ hybridization to determine the copy number of the gene (563-565). HER2 extracellular domain can be proteolitically cleaved and released into circulation and can be used for detection of primary and metastatic breast cancer by use of ELISA. Tumors that overexpress HER-2 develop a condition known as oncogene addiction (562). These types of cancer can be targeted in a receptor-dependent manner in even the most aggressive cancer types.

Triple negative breast cancer

Breast cancers can however become completely independent of the need for this receptor (as well as ER or PR) and become designated as triple negative breast cancer (TNBC) (51-514). This name denotes the lack of expression of these three receptors and are refractory to conventional chemotherapy and more difficult to approach therapeutically. There is currently fervent research being

conducted in order to develop new molecular based therapies towards this disease (51-54, 589). Breast cancer cell lines have helped in such endeavors and multiple TNBC cell lines (such as MDA-MB-231, BT549) are being used. Basal TNBC has high expression of cytokeratins 5/6 as well as EGFR and Ki67. Claudin-low express little to no expression of claudin-3, -4,and -7 proteins, which are required for cell attachment at tight junctions. These cells also express the epithelial marker E-cadherin (561,562). Both of these types are negative for mesenchymal marker N-cadherin (at resting conditions), although basal (MDA-MB-231) has high expression of cadherin-11, a breast cancer marker (561,562). The development of innovative drugs for TNBC is of utmost urgency. Below discussion of other novel breast cancer markers will be discussed.

Carbohydrate 15-3 (CA 15-3) and carcinoembryonic antigens (CEA)

Breast cancer that has metastasized becomes difficult to treat and Carbohydrate 15-3 (CA 15-3) and carcinoembryonic antigen (CEA) are used as markers of metastatic breast cancer (590,591). CEA is a glycosyl phosphatidyl inositol (GPI) cell surface anchored glycoprotein, which possess sialofucosylated selectin and E-selectin ligands, which is expressed in a vast majority of human colorectal, pancreatic, and breast carcinomas (590,592). These can serve as mediators of metastasis and enable dissemination into distal sites (590,592). The selectins are recognized by endothelial cells and facilitate cancer cell attachment to blood vessels and promote intravasation (590,592). Determination of CEA concentration is indicative of tumor size and nodal involvement and patients with levels >7.5

µg/L have a poorer prognosis than those within the normal range (<7.5ug/L) (590,592).

CA 15-3 peptides are shed from soluble forms of a mucin, known as MUC1, which is a transmembrane protein that is implicated in immunoevasion and metastasis. It has two subunits, which form a stable dimer (591). Release of CA 15-3 is mediated by 2 proteases ADMA17 and MT-MMP1 (591,593). This is heterogeneously expressed on the apical surface of epithelial cells and is overexpressed in 90% of breast cancer (591). There has been reports demonstrating a prognostic significance for CA 15-3 in patients that have HER2+ or HER2 overexpressing breast cancer (591,593). CA 15-3 may be of value in predicting disease relapse after surgery or in patients resistant to conventional treatments for this disease subtype (591,593). CA 15-3 seems to be a more reliable and reproducible marker of breast cancer compared to CEA, however the combination of CEA with CA 15-3 has been demonstrated to be a highly predictive marker of breast cancer (591,593).

BRCA1, BRCA2, and breast cancer

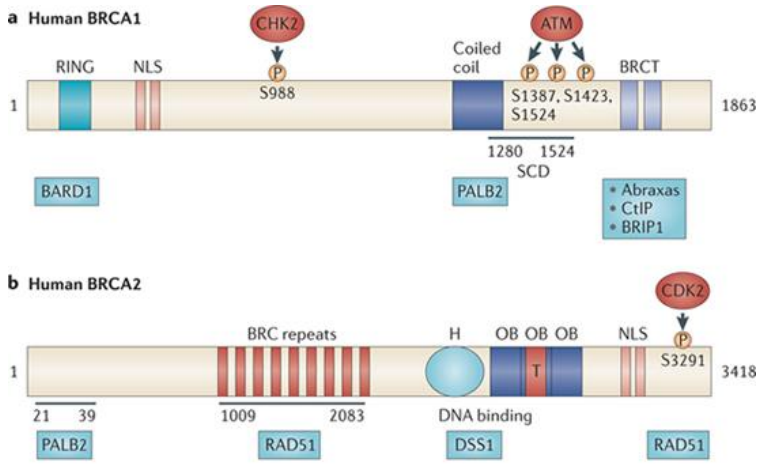

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Figure 38: BRCA1 and BRCA2. Structure and homology between Breast Cancer Associated (BRCA) 1 and 2.

Breast cancer also contains a direct hereditary component. Approximately 80% of the cases related to familial breast cancer are associated with the DNA repair tumor suppressor genes *BRCA1* and *BRCA2* (Figure 38) (594). BRCA1 and BRCA2 are important DNA repair enzymes that are stimulated by ATM (S988) and CHK2 (S187, S1423, S1524) by phosphorylation (594,595). BRCA proteins play a major role in transcriptional regulation, DNA repair, inhibiting cell proliferation and cell cycle arrest. BRCA1 combines with tumor suppressors and other signal transducers to form the BRCA1-associated genome surveillance complex (BASC) (594,595). This associates with RNA polymerase II as well as histone deacetylases via BRCA1 C-terminal domain. BRCA2 is also involved in the DNA damage response and involves BRCA2 directly interacting with RAD51

recombinase, through its BRC repeats, to promote strand invasion and subsequent recombination (594,595). The recognition of RAD51 to double stranded DNA requires BRCA2-BRCA1 complex along with BRCA partner PALB2 (partner and localizer of BRCA2) (594,595).

The frequency, spectrum, and mutations within *BRCA1* and *BRCA2* genes show considerable variation between geographic, demographic, and ethnic background, which emphasizes the role that heredity and environment, play in mutational rates and locations (596). Family history profiles can predict mutations in these genes mainly with characterizations of first-degree relatives with breast or ovarian cancer, along with young age of diagnosis, bilateral occurrence and increases number of affected relatives (596). Genetic counseling to identify *BRCA1 BRCA2* mutations is available and this may be able to serve as means for cancer prevention of at the very least prophylactic anticancer measures (594-596).

Kidney Cancer

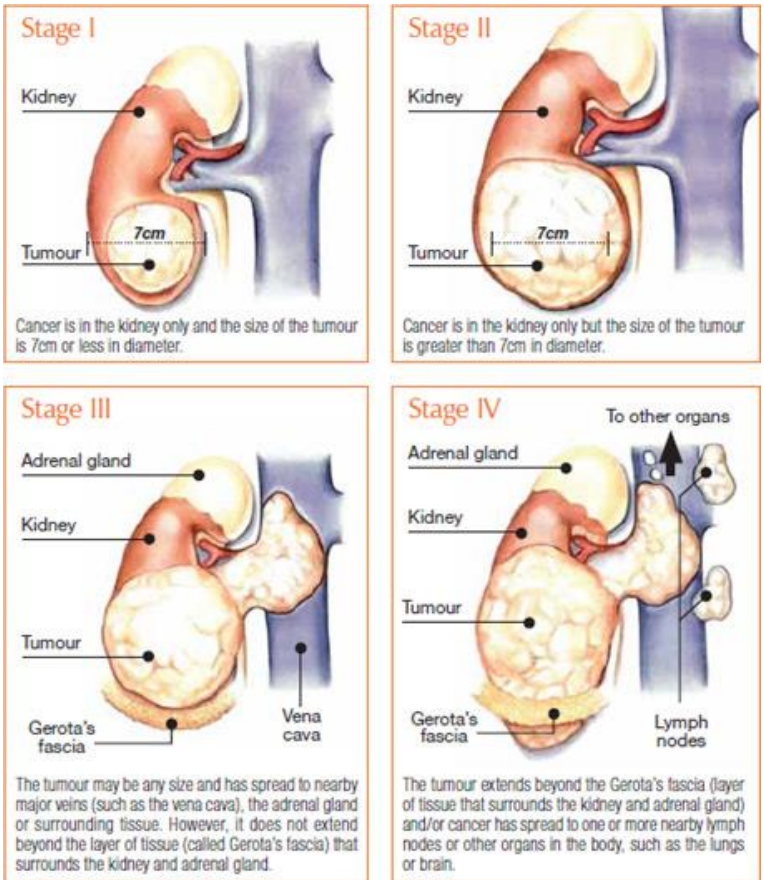
Kidney cancer classification

Kidney cancer represents the seventh most common type of cancer in the United States and it is estimated that in 2016 there will be 62,700 new cases in 2016 with over 14,000 of those resulting in cancer related death (20,21,597). The two most common types of kidney cancer are RCC (renal cell carcinoma) and

TCC (transitional cell carcinoma), with the latter sometimes being called urothelial cell carcinoma (597). RCC comprises approximately 80% of kidney cancer with the latter being mostly TCC. The overall 5 year survival rate in the united states in 73%, however kidney cancer (especially RCC) presents itself without any overt symptoms and early diagnosis is very difficult due to lack of good early stage markers (597). This 5 yr. survival rate also drops drastically if it spreads to the lymph nodes (65%) or metastasizes (i.e. pelvic abdominal cavity >12%) (597). Other less common types of kidney cancer include squamous cell carcinoma, juxtaglomerular cell tumor, angiomyolipoma, Bellini duct carcinoma, clear cell sarcoma, Wilm's tumor, mesoblastic nephroma, and mixed epithelial stromal tumor (or cystic nephroma); Squamous cell carcinoma is kidney cancer of the squamous cells. Juxtaglomerular cell tumors are tumors that arise from juxtaglomerular cells (597,598). These tumors often secrete high levels of the enzyme renin, which causes extreme cases of hypertension. Angiomyolipomas are tumors that have a high association rate with the disease tuberous sclerosis, which have a genetic component having mutant TSC ½, important tumor suppressors (597-599). Bellini duct carcinoma is very rare (1-3% of kidney cancer) and it is a tumor that arise from the papillary duct of the kidney. Clear cell sarcoma of the kidney, mesoblastic nephroma and Wilm's tumor are all pediatric associated malignancies, which occur very early in life (with mesoblastic nephromas being observed within the first 3 months of life) (599,600). Wilm's tumor is also referred to as a nephroblastoma and is quite heterogeneous,

containing blastemal cells (which have pluripotent propensity), mesenchymal cells, and epithelial cells (599,600). Wilm's tumors also have genetic components, which include mutations on Wilm's tumor genes (WT1 and WTX) and β-catenin (601,602).

Kidney cancer staging



https://pfizeroncology.com.au/understandingsomecancers/kidneycancer.aspx

Figure 39: Kidney cancer staging. Staging of kidney cancer follows the TNM classification system. Infiltration into lymph nodes and growth either within or beyond Gerota's fascia are assed to determine specific tumor stage and grade.

Kidney cancer is staged using the TNM system (as seen in breast and other cancer types) and has four stages (Figure 39) (603). At stage T1 there is a tumor that is between 4-7 cm within the kidney and it has not outgrown beyond the kidney (603). If it is 4 cm, it is T1a; if larger than four, it is T1b. At T2 stage, the tumor has grown beyond 7 cm but is still only within the kidney (603). If it has grown larger than 10 cm then it is classified stage T2b (if less than 10 then T2a). When the kidney tumor has grown into a major vein or tissue of the kidney, but has not infiltrated the adrenal gland or beyond Gerotias fascia (fibrous layer that surrounds the kidney) then the tumor is classified T3 (603). If the tumor is growing into the renal vein it is classified T3a (603). If the tumor is growing into the vena cava, it is classified as T3b and if this growth is near your chest than it is T3c (603). If the tumor has grown into the adrenal gland and/or beyond Gerotias fascia then it is labeled stage T4, and these type of tumors have a very high propensity for metastasis, especially to the pelvic and abdominal regions (603).

As stated, RCC represents the majority of RCC cases and represents 5% of adult epithelial cancers (20,21,603). RCC is a cancer that originates from the tubules of the kidney and clear cell carcinoma represents the most frequently observed subtype. Detecting these tumors early has been a substantial challenge; increased detection has been a consequence of increased screening, and advances in technology as these cancers are most often asymptomatic (604). The most common symptoms are abdominal and lower back pain and hematuria and manifestations are variable (603,604). RCC has also variable responses to

chemotherapy and radiation, and immunotherapies are promising (604,605). RCC responds the best to chemotherapies that target mTOR, angiogenesis (through VEGF and HIF-1α) and RTK inhibitors (i.e. EGFR inhibitors) such as the kinase inhibitor sorafenib and PDGFR and VEGFR inhibitor sunitinib (604,605). Other kinase inhibitors including pazopanib, axitinib, erlotinib, and cabozantinib (VEGFR) combined with mTOR inhibitors such as tesmirolimos and everolimous (analogs of rapamycin) and/or with bevacizumab (VEGF inhibitor) are among the most effective currently available chemotherapeutic regimens available for RCC today (606-610). Risk factors associated with RCC include environmental factors such as exposure to petroleum products, smoking (direct or secondhand), chlorinated solvents (such as trichloroethylene (TCE)), cadmium, lead, and asbestos (611-615). Diet and lifestyle also play a role as incidence is increased with obesity, insufficient water consumption, consume not enough water, high protein diets, high salt which leads to hypertension, and overuse of diuretic compounds (which can be drugs like clenbuterol, over the counter diuretics, or excessive caffeine consumption) (616-619). Kidney transplantation, history of kidney issues or dialysis use in the family, and HIV infection also increase the risk of RCC (603,605,607,617).

Certain genetic risk factors are associated with increased incidence of RCC, namely mutations in tumor suppressors VHL and TSC 1/2., which negatively regulate angiogenesis and mTOR signaling respectively (605). Indeed people who have con Hippel-Lindau disease have a 40% likelihood of developing RCC

at an early age, and its frequency is bilateral (75%) or multifocal (87%), with males at high risk (5 times) than females and African Americans have a higher incidence than Caucasians (328-330).

VHL and HIF-1 in RCC

Identification of the Von Hippel-Lindau (VHL) gene and elucidating its functions has helped substantially in developing RCC specific chemotherapy and has substantially enhanced our knowledge of RCC parthenogenesis. Loss of VHL is associated with VHL disease, which is a hereditary cancer syndrome that is characterized by the presentation of angiomatosis, phenochromocytoma, pancreatic and renal cysts, endolymphatic sac tumor, and hemangioblastomas (328-330). VHL is a tumor suppressor gene that is found on chromosome 3p25.3 and with affected families, the risk of RCC (and other cancers) is transmitted in an autosomal dominant manner (328-330). The VHL gene is a textbook example of the two-hit hypothesis. The two-hit (or Knudson hypothesis) states that cancer is caused by an accumulation of mutations, where two hits on a specific gene need to occur in order for a phenotype to emerge from these mutations (620). He demonstrated this with children with retinoblastomas who had inherited a mutated Rb gene and would have to incur another mutation or “hit” on the wild type allele for development of retinoblastoma (620). There are over 1500 germline and somatic mutations found in VHL disease, and individuals with VHL carry in their germline one wild type VHL allele, and one inactivated VHL allele (328-330). VHL manifests with full penetrance when the remaining wild type allele is inactivated

in a susceptible cell type (328-330). It has been demonstrated that inactivation of VHL is an early step in the pathogenesis of RCC in those individuals who have VHL disease. The VHL gene is mutated in approximately 50% of sporadic RCC and mutation is always observed in hereditary RCC (100%); therefore, the majority of RCC is linked by biallelic inactivation of VHL (328-330).

The VHL protein (pVHL) which is an important regulator of HIF1-α and plays an important role in drug metabolism and pathogenic defense in the intestinal tract. Under normoxic conditions, HIF1-α is hydroxylated by prolyl hydroxylases (PHD), and is recognized by pVHL (326-330). In hypoxic conditions, PHD is inactive, and HIF1-α accumulates, enabling it to translocate to the nucleus, associate with ARNT and express hypoxia inducible genes (326-330). When pVHL is mutated however, it does not recognize HIF1-α even when it is hydroxylated, which leads to constitutive activation of HIF1-α. This not only aberrantly upregulated genes involved in angiogenesis, but also glycolysis, glucose transport, pH regulation, cell cycle progression, and apoptosis (326-330).

VEGFR pathway and RCC

Constitutively active HIF1-α enhances VEGF and PDGF expression, which both play a substantial role in angiogenic signaling (315-325). Cancer cells can either directly activate angiogenesis through HIF1-α regulated pathways or by recruiting immune cells and exploiting the growth factors they release (315-325). There are several therapeutic strategies that have been designed to target

angiogenic pathways and these include VEGFR inhibitors such as vandetanib, and VEGF-R inhibitors (vatalanib) (315-318).

mTOR and RCC

Along with regulation translation, cell growth and ribosomal biogenesis, mTOR also regulates HIF1-α expression and particularly increases levels in individuals who have mutant pVHL (328-330). Patients who have RCC also have common mutations in the PTEN gene, which is a negative regulator of PI3K, further suggesting a link between mTOR necessity and angiogenesis (100,104). Inhibiting the mTOR pathway as well as PI3K/AkT pathway also inhibits growth and proliferation of endothelial cells and pericytes, which are crucial for angiogenesis (100,104). Furthermore, under excessive hypoxic stress a p53 upregulated gene REDD1 (Regulated in Development and DNA damage Response) (100,104). Upon its expression, it links protein p63 to upregulation of proapoptotic genes (PUMA, Noxa) and REDD1 directly inhibits mTOR (100,104,192,218-285). Together these three pathways converge on angiogenesis and excessive cell proliferation, which are hallmarks of cancer, and have a strong hereditary, as well as empirical and spontaneous component.

Rhabdomyosarcoma (RMS)

Rhabdomyosarcoma classification

Rhabdomyosarcoma (RMS) is the most prevalent soft tissue in children and adolescents, accounting for 5% of all pediatric cancers (621). RMS is a relatively rare cancer effecting 4.3 per 1 million children, with 350 estimated new cases being diagnosed in children and adolescents, and extremely rare in adults and persons over the age of 20 (621). The term rhabdomyosarcoma has a tripartite meaning: designating striated (rhabdo-) muscular tissue (myo-) and sarcoma, which is any cancer that is derived from mesenchymal stem cells and therefore mesenchymal in origin (621). RMS can originate from anywhere in the body and its etiology is elusive, with only some association with genetic inherited diseases such as neurofibromatosis type 1 and Li-Fraumeni cancer syndrome (621). RMS can occur either as a primary malignancy or as a component of a heterogeneous malignancy such as a teratoma, which is a tumor that contains cells that are derived from all three of the basic cell lineages: endoderm, mesoderm, and ectoderm (622).

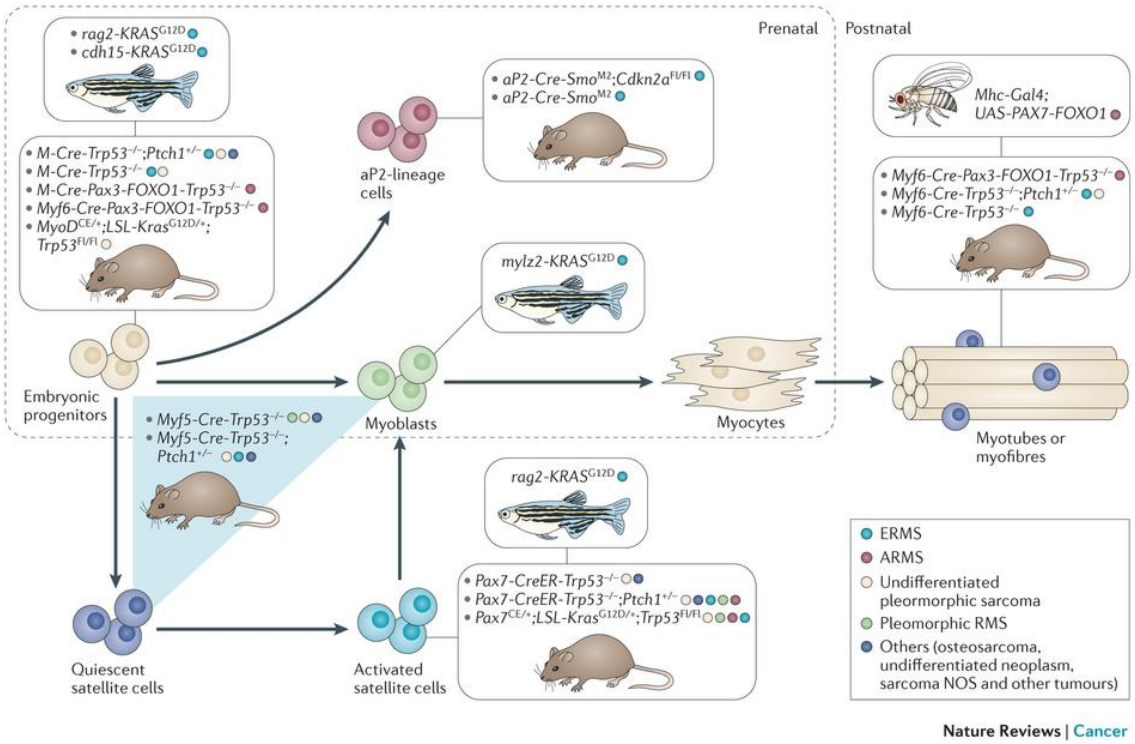


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Figure 40: Classification of rhabdomyosarcoma (RMS). There are two basic types: embryonal RMS (ERMS) and alveolar RMS (ARMS), with the latter having a very poor prognosis. Many animal models using genetic alterations (which both ARMS and ERMS have observed genetic components) can recapitulate the different types of RMS.

There are two basic types of RMS: embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS) (Figure 40) (621,622). There are also two smaller categories of pleomorphic RMS (PRMS) and sclerosis/spindle cell RMS (SRMS). ERMS however, has a much better prognosis and represents 60-70% of RMS cases (622,623). It has clear presentations with a characteristic pathology resembling “small round blue tumor cells” after H&E staining (623). Tumors are often present in the head and neck as well as the genitourinary track. ERMS can also be classified into small subdivisions based on cell morphology:

butyroid ERMS and spindle cell ERMS (622,623). Butyroid ERMS originates from mucosal lined organs such as found in the bladder, nasopharynx, and vaginal cavity and is found in infants less than a year old (622,623). It presents as a dense mass under an epithelial layer of tissue. SRMS is very similar to another sarcoma types of smooth muscle cells, leiomyosarcoma presenting with a fascicular, spindled and leiomyomatous growth pattern with notable myoblastic differentiation (623).

It represents 3% of RMS cases and presents commonly in the paratesticular regions. This type along with butyroid have very high survival rates with good prognoses (623,624). ARMS is the most malignant form of these RMS types and has a very poor prognosis, with less than a 10% survival rate. This type of RMS is characterized by densely packed, round cells that arrange is a fashion similar to pulmonary alveoli and forms in extremities, torso, and peritoneum (621-624). This type of cancer is very aggressive and most succumb to this disease (620-624).

Rhabdomyosarcoma staging

Diagnosis of RMS is contingent on the size, physiology, morphology, and metastatic propensity of the cancer, with ERMS and ARMS having stark differences for these categories (621-624). In order to stage RMS THE TNM stage and clinical group must be determined along with the type of RMS. Along with TNM and clinical group staging there is a risk stratification system that has

been established by the Intergroup Rhabdomyosarcoma Study Group (IRSG), which is highly predictive of outcome (625). TNM staging uses the classical tumor, lymph nodes and metastases characterization with other factors to determine the overall stage (625). Stage 1 the tumor has originated in a favorable area, which include the orbit, head or neck (with exception to parameningeal sites), genitourinary track, or bile ducts (621-625). Tumor size is not a factor in this staging and lymph node infiltration is possible, but metastatic lesions are not present. Stage II on the other hand has a tumor that has originates in an unfavorable region of the body, which include arms or legs, prostate, bladder, a parameningeal site, or other regions not lists as stage I criterion (621-625). Tumors are less than 5 cm and there are no metastases or lymph node infiltration. Stage III is similar to Stage II, with the only distinction being a tumor larger than 5 cm (with no lymph node infiltration or metastases) or the tumor is of similar size but has infiltrated adjacent lymph nodes. Stage 4 tumors have metastasized to distal organs such as lungs, bones, or bone marrow and the size of tumor and site of origin are irrelevant in this stage (621-625).

Stage	Organ	Tumor (T)	Size	Lymphnode (N)	Metastases (M)
I	Paratesticular, vaginal uterine	T1 or T2	a, b	N0, N1, Nx	M0
II	Bladder, prostate	T1 or T2	a	N0, Nx	M0
III	Bladder, prostate	T1 or T2	a	N1	M0
	b	N0, N1, Nx			
IV	All	T1 or T2	a, b	N0 or N1	M1

T1, tumor confined to site of origin (a, diameter <5 cm; b, diameter >5 cm).
T2, local infiltration, extension, or adherence (a, diameter <5 cm; b, diameter >5 cm).
N0, negative regional lymph nodes; N1, positive regional lymph nodes.
Nx, lymph nodes status unknown; M0, no distant metastases; M1, positive distant metastases.

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Figure 41: RMS staging. RMS is classified by the TNM classification system and by IRS clinical group classification. TNM staging considers the size, location, and the presence of lymph node infiltration and metastases. Clinical group classification considers the grade of the tumor after surgical resection.

Clinical group staging is based on the extent of the disease and the degree of efficacy in removal of the disease after initial surgery (Figure 41) (625). Group I includes children with localized RMS (with no lymph node infiltration or metastases) and the cancer has been consummately removed after surgery (625). This encompasses about 10-15% of children with RMS. Group II children have had all the visible cancer removed by surgery but remnants of the tumor

remain and can reside in the periphery of the original tumor site, in the lymph nodes or both (625). This comprises about 20% of RMS patients. Group III children have tumors that could not be completely removed by surgery and conspicuous parts of the tumor were left behind (625). Lymph node infiltration may be present but metastatic lesions are absent. This group represents approximately 50% of RMS patients (625). Group IV children the cancer has metastasized to the lung, bones, or bone marrow at the time of diagnosis and 15-20% of RMS patients are in this group (621-625).

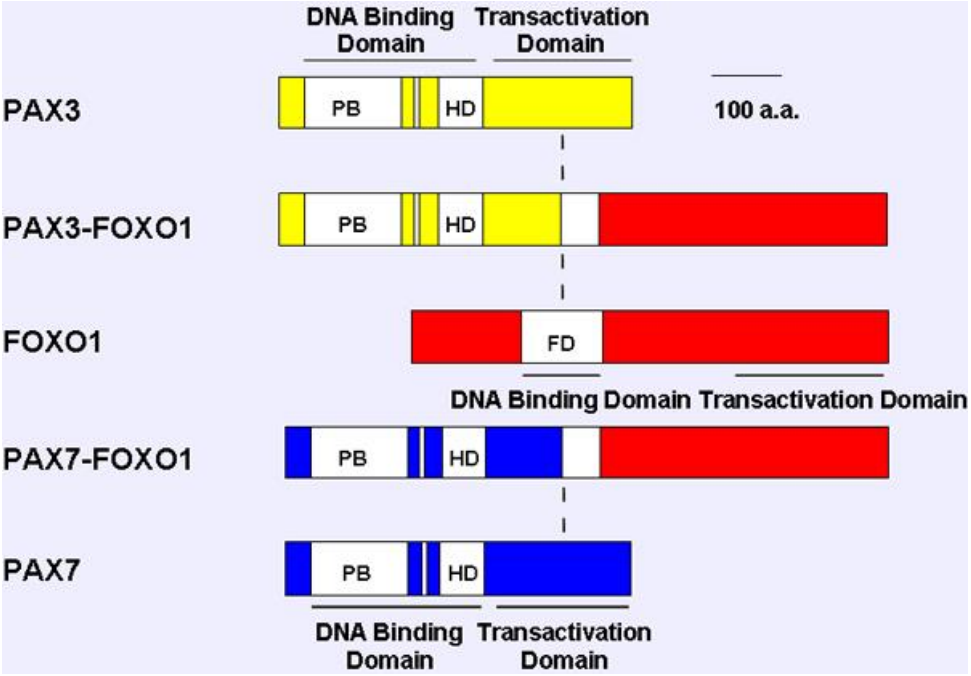
Risk group classification is determined by integrated information obtained from TNM and clinical group classification and is a determinant on the type and aggressiveness of the chemotherapeutic regimen (621-625). About 1 in 3 children are in the low risk group which include children with TNM stage 1 embryonal RMS that are in clinical groups 1, II, or III or children with stage 2 or 3 ERMS in clinical groups I or II (621-625). The intermediate risk group includes about 50% and is comprised of children with stage 2 or 3 ERMS or children with ARMS in stage 1, 2, or 3 (with no metastases present). The high-risk group includes children that have stage 4 RMS and metastases are present. Age correlates with some degree of tumor histology and primary site physiology (621-625). Patients who are over the age of 10 have a greater likelihood of ARMS whereas those younger than 10 are more likely to have ERMS (621-625). Treatment options available for RMS are very limited and include surgeries, radiation therapy, and a cocktail of very cytotoxic drugs (620-624). There is the VAC chemotherapeutic regimen, which

includes vinca alkaloid microtubule disruptor vincristine, actinomycin-D, which is a potent pan inhibitor of transcription, and the nitrogen mustard alkylation agent cyclophosphamide (621-624). Some drugs are replaced, such as ifosfamide, which is given concomitantly with mesna (sodium methane thiolate) to reduce the incidence of hemorrhagic cystitis and hematuria (621-624). A byproduct of cyclophosphamide metabolism is acrolein, which is toxic, and can be scavenged by mesna. Type I and Type II topoisomerase inhibitors irinotecan and etoposide are also used in conjunction with cyclophosphamide and vincristine (621-624). These are cytotoxic drugs that target all rapidly dividing cells.

Not only does RMS induce deleterious effects but the chemotherapies have detrimental effects later on in life (626). Indeed, there is a high incidence of adverse health outcomes (pulmonary, endocrine, cardiac, cognitive etc.) in those individuals who have overcome RMS (626). According to a St. Jude lifetime cohort study “at age 45 years, the estimated cumulative prevalence of any chronic health condition was 95.5% (95% CI, 94.8%-98.6%) and 80.5% (95%CI 73.0%-86.6%) for a serious/disabling or life-threatening condition” (626). Statistics, the ambiguous etiology, and paucity of efficacious chemotherapeutic regimens and procedures truly underscores the necessity to develop less cytotoxic drugs (621-626). There has been a resurgence in the endeavors to develop novel RMS chemotherapy and treatment. Recently two approaches involving lineage of origin and genetic studies which indicate that reactive oxygen species (ROS) inducing

drugs may be beneficial and this will be discussed in detail in the following sections (621-626).

PAX3-FOXO1 and RMS



http://atlasgeneticsoncology.org/Genes/GC_FOXO1.html (627)

Figure 42: PAX3-FOXO1 and PAX7-FOXO1. ARMS is distinguished by the presence of the Pax 3-FOXO1 fusion gene product. This translocation is observed in 80% of ARMS cases and involves chromosomal translocation associated with t(2;13)(q35;q14) and t(1;13)(p36;q14), involving the 5' DNA-binding region of PAX and the transactivation domain at the 3' end of FKHR. The PAX7/FKHR fusion gene product t(1;3(q36;q14) is also observed but only in 25% of translocation-positive patients

Comparative Genomic Hybridization (CGH) analysis has revealed that all RMS types have specific acquisition or loss of chromosomes and exhibit chromosomal abnormalities (627,628). ERMS frequently exhibits alterations is whole chromosomes, whereas ARMS is characterized by the presence of regions

of genomic amplification. The genes most of which have amplifications are *CDK4, MYCN, GLI, MDM2, FGFR1*, and *FGFR4*, which correspond to chromosomal regions 12q13.3-q14.1 (627-631). Most genes that are aberrantly expressed due to chromosomal abnormalities are oncogenes, which increase cell proliferation or inhibit tumor suppressors. ARMS tends to have less copy number variants than ERMS, and chromosomal amplification of gene *TYROBP, LFRN3*, and *HCST* were observed in ERMS but not ARMS (627-631). Along with regional genomic amplification, chromosomal translocations are also frequently observed in ARMS. These involved the myogenic paired box transcription factor PAX3 and the transcription factor FOXO1 (FKHR), a member of the forkhead/HNF-3 transcription factor family (632,633). This translocation is observed in 80% of ARMS cases and involves chromosomal translocation associated with t(2;13)(q35;q14) and t(1;13)(p36;q14) (Figure 42) (632,633). The chromosomal translocations involve the 5' DNA-binding region of PAX and the transactivation domain at the 3' end of FKHR (632,633). It is estimated that 75% of these chromosomal rearrangements translocate the PAX3 (2q25) gene to FKHR (13q14) with the other 25% being associated with a PAX7/FKHR fusion gene product t(1;3(q36;q14) (632,633). The other 20% of ARMS patients lack either translocation variant (designated *PAX* gene fusion negative or PFN) and these pose a challenge to detect (632,633). Risk stratification analysis has demonstrated that PAX3/FKHR status is a beneficial prognosis marker, whereas evidence for the PAX7 derivative is equivocal and inconclusive (632,633).

Recently a paper was published in *Genes and Development*, which addressed the lineage or origin concept in ARMS (634). They used conditional genetic mouse models of ARMS to recapitulate the PAX3:FOXO1 fusion genes and inactivate p53 and demonstrated that lineage of origin had a substantial impact on the histopathology and morphology of the tumor and its sensitivity to chemotherapeutic agents (634). They concluded that the p53 is often mutated in RMS and that these tumors that express the PAX3:FOXO1 fusion gene forms sarcoma for satellite cells that are distinctive from any other myogenic lineage and it was PAX3, not PAX7, fused with FOXO1 along with p53 mutation in these mouse models that congruently recapitulated the human ARMS phenotype (634). PAX3:FOXO1 was also demonstrated to be epigenetically regulated and that DNA methyltransferase inhibitor 5'Aza 2'deoxycytidine or etinostat (HDAC inhibitor) showed a significant decrease in PAX3:FOXO1 mRNA and protein expression, with a more pronounced effect with etinostat (634). They also observed decreased tumor growth in these mouse models with etinostat treatment (634). They successfully demonstrated the therapeutic potential that HDAC inhibitors play in a human ARMS model; however, the exact mechanism was not elucidated.

ROS (reactive oxygen species), sources of ROS, and ROS scavengers

In humans, as well as mammals, the generation of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) is a natural consequence of aerobic metabolism (Figure 43) (635). Cells have evolved mechanisms to reduce the concentration of these species as many can be deleterious to the cell. These

include non-radical species and radical species. Non radical species include hydrogen peroxide (H_2O_2) and organ peroxides (ROOH), superoxide ($O_{2^{-\cdot}}$) singlet Oxygen ($O_{2)}$), ozone (O_3), alkoxyl and peroxyl radicals ($RO^{\cdot}$ $ROO^{\cdot}$) hypochloride (HOCl), peroxynitrite ($ONO^{-\cdot}$), nitrososperoxycarbpnate ($O{=}NOOCO_2^-$), nitrocarbonate ($O_2NOCO_2^-$), nitrogen dioxide (N_2O_2), nitronium ions (NO_2^+) which can cause lipid peroxidation and modification to DNA (such as 8-oxo-guanine) that impair cellular homeostasis (635-637). These enzymes include super oxide dismutases (SOD), catalases, glutathione synthesis pathways, glutathione-S-transferase, and NRF2 (through antioxidant response elements) (635-637). SOD uses a disproportionation reaction (a concomitant oxidation and reduction of a species to generate two products) to generate hydrogen peroxide which is subsequently neutralizes to water and oxygen by another round of SOD and catalase.

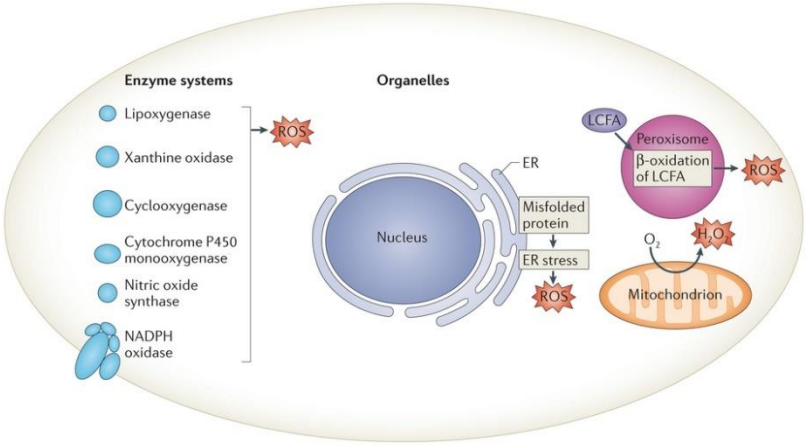


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Figure 43: Sources of reactive oxygen species (ROS). Mitochondiral respiration, NADPH oxidase, 5-lipoxygenase (are sources of ROS in normal and transformed cells. Xanthine oxidase, eNOS (endothelial nitric oxide synthase) uncoupling and mitochondrial disfunction also increase ROS production. In muscle derived cancers ROS levels are substantially elevated and cancer cells upregulate expression of antioxidant enzymes including glutathione S-tranferase, thioredoxins, glutathione peroxidases, and peridoxins.

Cancer cells exploit the role of peroxide as a bonafide signaling molecule and whether this molecule signals survival or death pathways is determinant on the fine-tuned gradient of hydrogen peroxide that is generated in the cell (635-637). Hydrogen peroxide, as well as other peroxides and other peroxidase lipids can be neutralized by thioredoxins, peridoxins, and glutathione peroxidases. The latter are unique in the fact that they contain a noncanonical encoding amino acid at its active center, selenocysteine (635-637). The amino acid acts very similarly to a cysteine in that it generates a very reactive selenite just as cysteine can form reactive thiolates. However, selenates are much more reactive and have very efficient enzyme kinetics (635-637). The mitochondria are the main generator of ROS, which are byproducts of oxidative pathways such as oxidative

phosphorylation and β-oxidation (635-637). Other enzymes such as NADPH oxidase (involved in pathogen defense and signaling mechanisms), xanthine oxidase (converts hypoxanthine into xanthine for purine salvage pathway), and heme oxygeneases generate ROS and these species are neutralized by antioxidant chemicals such as glutathione (gamma glutamylcysteinyl glycine), S-adenosylmethionine, and N-acetyl cysteine (635-637). ROS also plays an important role in innate immunity and signaling. Another enzyme that deliberately generates ROS is nitric oxide synthase (NOS), which generates the reactive radical messenger nitric oxide (635-637). Reactive thiyl species are generated by these reduced sulfhydryl groups reacting with ROS and RNS (638). This stimulates guanylate cyclase, producing cyclic guanosine monophosphate. This activates protein kinase G (PKG) that induces reuptake of calcium and activation of calcium activated potassium channels (638). The fall in calcium disables myosin light-chain kinase (MLCK) from phosphorylating myosin. This promotes vasodilation in endothelial cells and pathogenic defense in macrophages (470,471,481,482,638).

Cancer and ROS

ROS levels in cancer cells exceed normal physiological thresholds. This is due to metabolic shifts to aerobic glycolysis, hypoxic stress, increased stress from increased anabolism, and stress from chemotherapeutic drugs (whether that be a direct or ancillary consequence of the drug). Many cancers also arise from chronic inflammation, irritation or infection that induce ROS and tumor cells recruit

immune cells such as macrophages that secrete TNFα, which can generate ROS (470,471,481,482,638). Some cancers derived from cells that undergo high levels of oxidative metabolism are especially prone to ROS. Pancreatic cancer and RMS both have high levels of ROS that are just marginally under the critical threshold that induces ROS-dependent cell death in these cells. Thus chemotherapeutic drugs that induce ROS are highly effective against these tumors (635-638). ROS-inducing anticancer agents include cytotoxic drugs that induce to excessive stress (such as DNA alkylating agents daunorubicin and doxorubicin, and microtubule disrupting agents like Taxol) and compounds that target mitochondria (635-638). Some compounds also indirectly induce ROS by inhibiting the synthesis or reducing the concentration of cellular antioxidants. Buthionine sulphoximine inhibits glutathione (GSH) synthesis by inhibiting the enzyme gamma glutamyl cysteine synthase, Imexon and DEM (diethyl malonate) deplete intracellular levels of GSH, and 2-methoxyestradiol inhibit SOD directly and tertamethylmolybdate acts as copper sink, needed for SOD (639). There are also natural products (phytochemicals) and analogs that have ROS-inducing anticancer activity. The compound piperlongumine (PL) from the Long pepper and nimbolide, a terpenoid lactone, are ROS-inducing agents (640,641). Our laboratory investigated several ROS inducing compounds, including curcumin, triterpenoids (betulinic acid, CDDO-Me), celastrol, and the isothiocyanate PEITC (642-648). We recently reported that PEITC induced apoptosis, decreased cell proliferation, and inhibited migration in pancreatic cancer cells all of these responses were ameliorated by

pretreatment with the antioxidant glutathione (648). PEITC downregulated Sp (specificity protein) transcription factors in an ROS-dependent manner (648). This effect was initiated by ROS-dependent downregulation of c-Myc in an epigenetic fashion, which led to a decrease in miRNAs 17, 20, and 27a (Figure 44) (648). These miRNAs are negative regulators of ZBTB proteins which are induced (by ROS) resulting in downregulating of pro-oncogenic Sp-regulated genes (648).

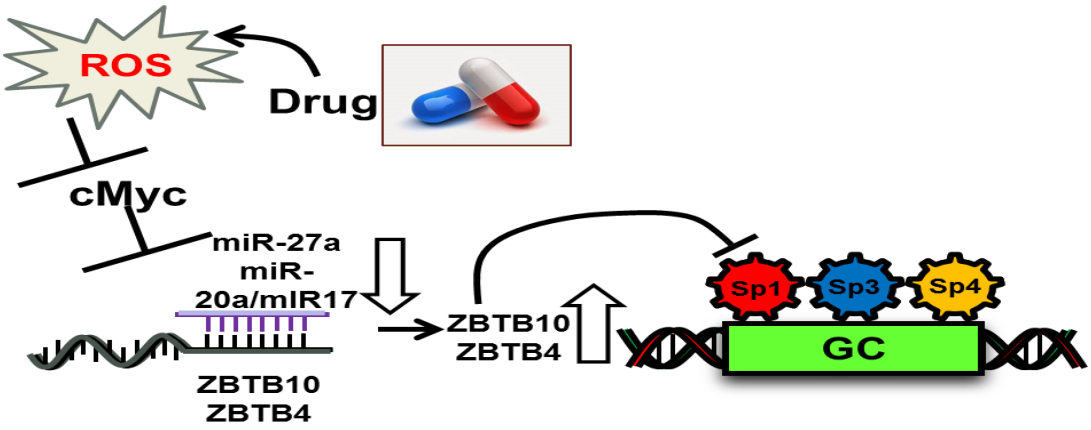

Figure 44: Mechanism of ROS anticancer activity. Drugs that induce ROS inhibit cancer growth by targeting cMyc. This downregulation of cMyc leads to downregulation of miR-27a, miR-20a, and miR-17. This leads to an increase in ZBTB 10 and 4 expression. ZBTB proteins negatively regulate Sp proteins and inhibit their transactivational activities. Drugs such as isothiocyanates, NSAIDs, triterpenoids, curcumin, artemisinin, and HDAC inhibitors have been demonstrated to induce ROS.

RMS and ROS: histone deacetylases (HDACs) and HDAC inhibitors

RMS has also recently been shown to be sensitive to ROS inducing agents (635,626). The anti-malaria drug artemisinin, induced apoptosis in RMS and had antitumor effects (647). The drug curcumin has also been demonstrated to inhibit RMS tumorigenesis by inhibiting the mTOR pathway (649). In Cancer cell, a

critical paper was published that demonstrated the effects of a myriad of ROS inducing agents and their anticancer effects on a multiple types of tumors (650). They used genomic analysis to demonstrate that ERMS have high copy number variants associated with genes, namely those involved in the RAS/NF1 pathways, which is associated with increased risk (650). They developed orthotopic xenografts and through high throughput screening of primary cultures concluded that oxidative stress was a significant pathways that had therapeutic

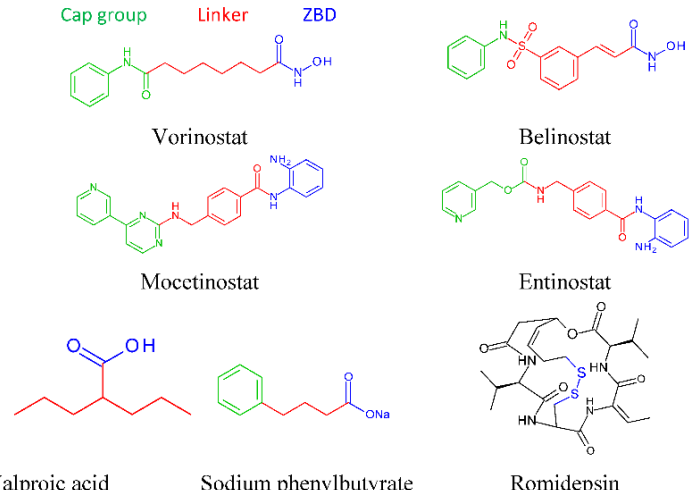

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Figure 45:HDAC inhibitors (HDACi). All HDACi consist of a capping group, an aliphatic linker, and a functional group which interacts with the Zn2+ binding pocket and inhibits the HDAC. B) There are 4 classes of HDAC inhibitors: hydoraxmic acid derivatives (vorinostate and belinostat), aliphatic acids (magnesium valproate, sodium phenylbutyrate, depsipeptides (rhomidepsin), and benzimides (mocetinostate and etinostat). They have varying specificity and potency against cancer and their efficacy is tissue specific. HDAC inhibitors have shown great promise in hematological malignancies and vorinstat is FDA approved for cutaneous T-cell lymphoma.

relevance in treating ERMS (as well as other tumor types such as pancreatic and ovarian) (650). One of these agents was a histone deacetylase inhibitor (HDACi)

panobinostat (LBH589) (650). HDACi are a class of compounds that inhibit the enzymes known as histone deacetylases (HDAC) (Figure 48) (650). These enzymes deacetylate lysine residues on histone tails (650). HDACi (illustrated in figure 45) have long been of interest in cancer research as chemotherapeutic agents. While their anticarcinogenic effects have been very promising in hematological malignancies, their efficacy in solid tumors is modest and they have been considered only as adjuvants to existing more cytotoxic chemotherapeutic regimens. Vorinostat has been FDA approved for treatment of cutaneous and peripheral T-cell lymphoma (CLTL) and vorinostat is in clinical trials for treating GBM as an adjuvant with temozolomide (651). Rhomidepsin has also been approved for CTCL and is clinical trials for T-cell lymphomas. Another pan-HDACi that has been used extensively in research and clinical setting id the drug belinostat. In addition, it has been approved for treatment of peripheral T-cell lymphoma (PTCL) (652). Panobinostat is a pan-HDACi that is sold under the trade name Raydak by Novartis and on February 2015, it received FDA approval for treating multiple myeloma in patients that had already received two other treatments that included bortezomib and thalidomide or one of its analogs (lenalidomide) (653). It is currently in clinical trials for Hodgkin's Lymphoma, CTCL, myelodysplastic syndromes, breast cancer, and chronic myelomonocytic leukemia (CMML) and prostate cancer (653-656). Recently Panobinostat has been shown to selectively target TNBC breast cancer and reduce hypoxia induced cisplatin resistance in NSCLC by destabilizing the HIF1-α (654). Panobinostat

also induces autophagic cell death in liver cancer (657). Many of these effects of panobinostat and other HDACi may have an underlying oxidative stress component and in this thesis, the research of panobinostat as an ROS inducing agent that specifically targets RMS (both ERMS and ARMS) cancer cells and the results will be reported in chapter IV.

Colorectal Cancer (CRC)

CRC classification

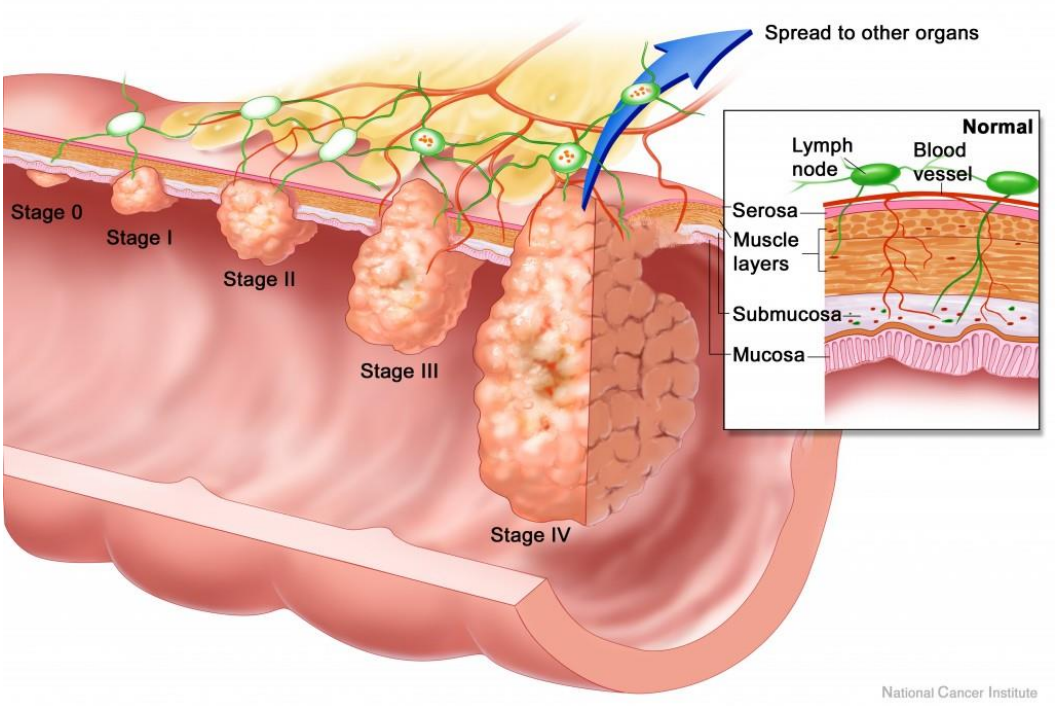
Colon or colorectal cancer (CRC) is the second and third most commonly diagnosed cancer in women and men respectively and more than one million people worldwide are diagnosed with tumors in the colon every year and recent molecular studies demonstrate that tumors in colon and the rectum are the same type (20,21). Colon or colorectal Thus CRC is the generic term for several types of cancer that arise in the colon and rectum, including adenocarcinoma, carcinoid and stroma tumors, lymphoma and sarcoma (20,21,658). Adenocarcinomas originate from the gland cells that make the mucus of the colon and rectum and are epithelial in origin (658). Carcinoid and stromal tumors originate from specialized hormone-producing cells and barrier-forming cells respectively and they can be found in other regions of the gastrointestinal tract (659). Lymphomas originate from cells in the lymph nodes associated with the intestinal tract and

sarcomas originate from connective tissues and cells of mesenchymal origin such as muscles, blood vessels (658,659).

CRC: lifestyle and diet

Factors that directly influence CRC include diet (high fat, alcohol, and low fiber, high consumption of red and processed meats), sedentary lifestyle, smoking, and obesity (658,659). High fat and low fiber consumption lead to a state of constant inflammation within the colon that promotes cancer and the formation of polyps. Obesity exacerbates this condition and so does lack of exercise. Smoking release a myriad of carcinogenic compounds, the most notable being benzo (*a*) pyrene (32,658,659). Red meat and processed meat have high levels of nitrites, which can form nitrosamines, which are known carcinogens. Consumption of barbequed meat also leads to the of carcinogenic polyaromatic heterocyclic amines such as PhIP (2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (660). Adenocarcinomas are the most commonly diagnosed colon cancers and account for 95% of all colon cancer cases (21,660). They arise from adenomatous polyps such as adenoma formed in the innermost lining of the colon that eventually grow past the wall and into the underlying tissue (660). When cancer cells invade the blood and lymphatic vessels, they metastasize to other parts of the body, favoring tissues such as the liver by traveling the portal vein (51-54,660).

CRC staging and chemotherapeutic regimens



http://fightcolorectalcancer.org/fightcrc-fightit/diagnosis-staging/ (661)

Figure 46: Stages of colorectal cancer. Stage 1 tumors are polyps that have infiltrated the submucosa or the muscularis propria, designated T1 or T2, and no lymph node invasion or metastases are observed yet. Stage 2 tumors can be broken down into three different categories: Stage IIa, Stage IIb, and Stage IIc. Stage IIa the tumor has grown into the outermost layers of the colon wall but has not grown through them. Stage IIb the tumor has both grown into and through the colon wall. With both Stage IIa and Stage IIb, the tumor has yet to make contact or attach to any nearby organs, which is in contract to stage IIc, where the tumor has made contact/attached to a nearby tissue or organ. Most cases the tumor has not spread to the lymph nodes or metastasized at any of these stages. In stage III the tumor has grown through the mucosa into the submucosa and into the muscularis propria, or through the visceral peritoneum. Stage IV colon cancer indicates that it has metastasized to other tissues, such as the liver or the lungs.

Colon cancer can be categorized into four different stages with each stage representing an increased aggressiveness and likelihood of metastasis with the disease (Figure 46) (661). After carcinogenic transformation, neoplastic cells form a high-grade dysplasia, which is either a hyperplastic or inflammatory polyp. This

polyp is termed carcinoma *in situ* (or an intramucosal carcinoma) which is a superficial non-invasive pre-cancer lesion. This can be designated as stage 0. These polyps can be removed successfully without any future disease recurrence or potential of cancer later on in life with an endoscopic method called a polypectomy. Stage 1 tumors are polyps that have infiltrated the submucosa or the muscularis propria, designated T1 or T2, and have no lymph node invasion or metastases (661). Stage 2 tumors can be broken down into three different categories: Stage IIa, Stage IIb, and Stage IIc (661). In Stage IIa the tumor has grown into the outermost layers of the colon wall but has not grown through them where in Stage IIb the tumor has both grown into and through the colon wall (661). With both Stage IIa and Stage IIb, the tumor has yet to make contact or attach to any nearby organs, which is in contrast to stage IIc, where the tumor has made contact/attached to a nearby tissue or organ. Most cases Stage 2 tumors have not spread to the lymph nodes or metastasized at any of these stages. In stage III the tumor has grown through the mucosa into the submucosa and into the muscularis propria, or through the visceral peritoneum, and it has spread to 1-3, or 4-6 lymph nodes (661). These stages again can be ranked as Stage III A, Stage IIIB or Stage IIIC, depending on its level of penetration and how many lymph nodes have been infiltrated (661). Stage IV colon cancer indicates that it has metastasized to other tissues, such as the liver or the lungs (661). Surgery is the initial treatment for early stage CRC and a complete removal of the tumor without disease remission can be accomplished (661). This type of therapy however, is

not fully efficacious for stage IV patients since the metastases have already colonized sites at distal organs and systemic cytotoxic chemotherapeutic regimens are required to fully remove the cancer (661). Chemotherapeutic drugs often used to treat CRC, especially in advanced stages and these include the K-ras inhibitor panitumumab, platinum based DNA crosslinking drug oxiplatin, toxic nucleotide analog capecitabine, thymidylate synthase inhibitor 5-FU and folate analogs (leucovorin), EGFR inhibitor cetuximab, VEGF signaling inhibitors bevacizumab aflibercept and ramucirumab, and the DNA topoisomerase I inhibitor irinotecan (662-669).

CRC: hereditary component

Hereditary colon cancers are usually initiated by loss of heterozygosity (LOH) at the genomic loci that encode or regulate a tumor suppressor protein product (670). There are two major types of hereditary CRC: familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) (670,671). FAP and familial colorectal polyposis (Gardner syndrome) have mutations in the gene adenomatous polyposis coli (APC) which is a powerful tumor suppressor that suppresses Wnt signaling (672). Wnt signaling stimulates nuclear localization of the transcription factor β-catenin, which is a powerful proliferative factor and driver of CRC (672). HNPCC is caused by defects in DNA repair proteins involved in MMR (mismatch repair) such as MSH2, MLH1, MSH6/GTBP, and PMS2 (411-416,673).

CRC: Crohn's disease and IBD

People who have Crohn's disease or any other kind of inflammatory bowel disease or also at greater risk for CRC (674). Meta-analysis studies indicate that the risk for colon cancer increases with the duration and extent of inflammation in ulcerative colitis but not in Crone's disease, which coincides with the higher incidence of cancer in patients who have ulcerative colitis (674-676). More than 20% IBD result in CRC within 30 years of the onset of disease and these cases are often referred to as colitis-associated cancer (CAC), a colon cancer subtype that has a mortality rate higher than 50% (674-676). CAC and non-CAC cancers share similar mechanisms of parthenogenesis, which can be summarized as the adenoma-carcinoma sequence (674-676).

The adenoma-carcinoma sequence

The concept of carcinogenesis being a multistep process was first applied in the development of CRC in 1990 (677). The adenoma-carcinoma sequence model (vogelgram) was later proposed to describe the consecutive genetic changes, which progress over several years and eventually lead to colon carcinoma (Figure 47) (677). Environmental factors such as mutagens and ROS induce DNA damage and initiate the formation of aberrant crypt foci (ACF) (677). Mutations in APC, Wnt signaling pathways are early events that trigger the outgrowth of ACF to hyperplastic lesions projecting above the colorectal mucosa known as polyps (677). As stated earlier β-catenin, is a potent driver of cell

proliferation that mediates the effects of Wnt signaling on stem cell proliferation, differentiation and shedding of crypt cells into the lumen (671,672,677).

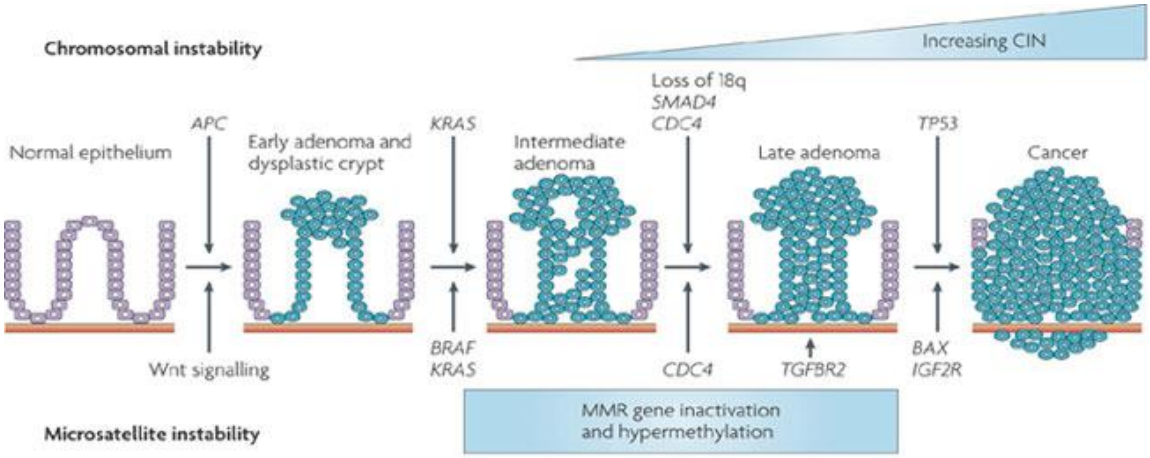


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Figure 47: The adenoma-carcinoma sequence. Environmental carcinogens serve as initiators and then mutations in APC lead to promotion of these cells that grow into an early adenomas. Mutations in Kras then excelerate the growth of these benign neoplasms. Carcinomas of the colon are also like other tumors in which they are loss of function diseases, which include loss of tumor suppressors and loss of pro-apoptotic signaling by TGFβRII. Activation of oncogenes and inactivation of tumor suppressors contribute to uncontrolled cell proliferation and genetic instability which results in an invasive and metastatic colon carcinoma.

Other components of the Wnt signaling pathway include APC, GSK3β, CK 1, and Axin and mutations in either APC (loss of function) or β-catenin (gain of function) enhance the formation of ACF and polyps, with APC being a very large gene with multiple regions for spontaneous inactivating mutations to occur (671,672,677). Not all hyperplastic polyps become cancerous, however, as only polyps from the glandular epithelium or adenomatous polyps development into adenomas. The formation of adenomas is characterized by the upregulation of

oncogenes such as K-ras (80% of CRC patients), NFκB, Stat3, and B-Raf (677,678). The transition from adenoma to carcinoma requires inflammatory conditions, which is associated with the upregulation of cyclooxygenase 2 (COX-2) (459-466,677). Carcinomas of the colon are also like other tumors in which they are loss of function diseases, which include loss of tumor suppressors and loss of pro-apoptotic signaling by TGFβRII (678,679). Activation of oncogenes and inactivation of tumor suppressors contribute to uncontrolled cell proliferation and genetic instability which results in an invasive and metastatic colon carcinoma (676-678).

Pancreatic Cancer

Pancreatic cancer classification

It is estimated by the American cancer society that about 53,070 individuals will be diagnosed with pancreatic cancer and of these over 41,000 will die from the disease (20,21,680). It is the fourth leading cause of cancer related death and 7% of cancer related death are attributed to pancreatic cancer (20,21,680). Pancreatic cancer is a debilitating disease with one of the lowest 5-year survival rates of any cancer at less than 6%. It is predicted to be the second deadliest malignancy in the USA by 2020 (680). While the survival rates for other cancers, especially gastrointestinal cancers, has increased substantially in the last 30 years the progress with pancreatic cancer has been rather stagnant. Traditionally

surgery is the best strategy for removal of the cancer with chemotherapeutic strategies having little efficacy and usually patents after a long battle will only have palliative treatment (680,681). Even when surgery is successful, over 80% of patients have disease relapse and only 10-15% of pancreatic tumors are considered resectable at diagnosis (680,681). The high mortality rate and lack of truly efficacious treatment is due to the paucity of early stage molecular markers available for pancreatic cancer. Preclinical evidence suggests pancreatic cancer is a systemic disease, which suggests potential ameliorative effects through pre-emptive systemic therapy (680,681). Currently therapeutic regimens for metastatic pancreatic cancer are FOLFIRINOX (folinic acid, 5-FU, irinotecan, and oxiplatin) and nab-paclitaxel plus gemcitabine, which have modest efficacy at best (682). Therefore, there is a high necessity for not only the discovery of novel biomarkers, but innovation of more efficacious therapy.

Pancreatic cancer are all malignancies, which originate in the body of the pancreas, which is composed of the endocrine component called the islets of Langerhans (680,681,683). These cells produce hormones such as glucagon, insulin, somatostatin, and enzymes for digestion such as trypsin and lipases, which are secreted into the duodenum (680,681,683). Tumors originating from endocrine glands are rare and are designated neuroendocrine tumors with 85% of pancreatic cancer is pancreatic adenocarcinoma (680,681,683). These can also be classified as exocrine tumors and can be subcategorized into different subtypes. After adenocarcinoma, acinar cell carcinoma of the pancreas is the

next common (680,681,683). They can over-produce certain enzymes, mainly lipases and present with subcutaneous fat necrosis, polyarthritis and eosinophilia (the so call Schmidt Triad) (684). Cystadenocarcinomas account for 1% of all exocrine tumors and are malignant cystadenomas. Pancreatic cancer associated with children is pancreatoblastoma (680,681,683,684). Other exocrine tumors present as pancreatic mucinous cystic neoplasms and they appear as mucin filled cysts (683,684). All these exocrine tumors are formed in the ductal epithelium and are referred to collectively as pancreatic ductal carcinoma.

Pancreatic intraepithelial neoplasia (PanIN) staging

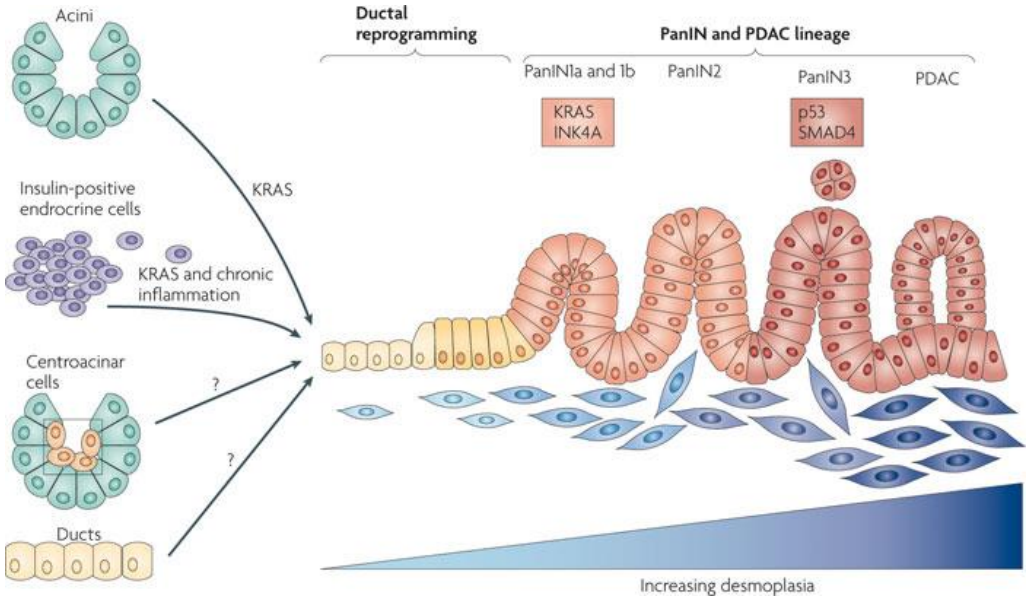

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Figure 48: Pancreatic Intraepithelial Neoplasia (PanIN) staging. The pathological features of pancreatic ductal adenocarcinomas are categorized by the pancreatic intraepithelial neoplasia (PanIN) nomenclature system (PanIN-1 to 3) which depicts the consecutive changes in morphology and histology. These stages are also associated with changes in gene expression and upregulation of proteins. For example, in PanIN1 you have constitutive Kras activation and gene amplification of HER2. In later stage PanIN you have loss of p16 (PanIN-2) followed by loss of p53, SMAD4, and BRCA2 (PanIN-3).

The epithelium of the pancreatic duct consists of cuboidal or low-columnar cells with amphiphilic cytoplasm and occasionally this epithelium is replaced with mature squamous cells, which form transitional metaplasias and they are not necessarily precancerous (685). In many cases, this is accompanied by atypia, or other abnormalities, which initiate cancer (685). The pathological features of pancreatic ductal adenocarcinomas are categorized by the pancreatic intraepithelial neoplasia (PanIN) nomenclature system (PanIN-1 to 3) which depicts the consecutive changes in morphology and histology (Figure 48) (685). These lesions exhibit varying levels of cellular atypia and are considered precursors of metastatic PDAC that develops over many years (685).

The initial stage of these lesions is PanIN-1A, which refers to epithelium that has normal appearance and is almost histologically congruent to normal pancreatic epithelium (685). PanIN-1B is histologically similar to PanIN-1A except for the presentation of micropapillary and basally pseudostratified structure (685). PanIN-2 has large nuclei, which are crowded, loss of cell polarity, hyperchromatism, and the majority of the cells are surprising quiescent (685). PanIN-3 lesions have morphology similar to goblet cells with mucinous cytoplasm orientated towards the basement membrane, large nucleoli and other nuclear irregularities (685). These lesions also will sporadically release necrotic epithelial cells into the lumen. These cells undergo rapid mitosis and can be classified as carcinoma in situ, which is Stage 0.

Pancreatic cancer staging

Stage	Characteristics	Median Survival (Months)
IA	Tumor < 2 cm and limited to pancreas No lymph node involvement	24.1
IB	Tumor > 2 cm and limited to pancreas No lymph node involvement	20.6
IIA	Tumor extends beyond the pancreas (no superior mesenteric artery or celiac axis involvement) No lymph node involvement	15.4
IIB	Any size tumor with regional lymph node involvement	12.7
III	Tumor involves the superior mesenteric artery or celiac axis ± lymph node involvement No distance metastasis	10.6
IV	Any size tumor ± lymph node involvement Distance metastasis	4.5

http://geekymedics.com/pancreatic-cancer/ (686)

Figure 49: Pancreatic cancer staging. TNM classification system for pancreatic cancer.

Pancreatic cancer uses the same TNM classification as other cancer types (Figure 49) (686). Stage 0 dysplasia is limited to the top layer of the pancreatic duct (Tis), and the carcinoma is contained within the pancreas. Stage 1 refers to pancreatic tumors that are grown locally and are less than 2 cm across (T1) (686). Stage1 T2 indicates greater than 2 cm and in either case there is no spreading (N0, M0) (686). Stage II indicates infiltration of the tumor outside the pancreatic parenchyma with some infiltrating lymph nodes but no metastases (T3) (686). Stage III is similar to stage II, to where the tumor has grown larger and is proximal to blood vessels and nerves (T4) (686). Stage IV pancreatic cancer indicates metastasis (M1 or greater) and this is the most devastating stage of this disease with a very desolate prognosis (686). Stage I and Stage II pancreatic tumors can be treated by using the Whipple procedure, which is a procedure involving a

pancreaticduodenectomy (686,687). This involves removal of the head of the pancreas, lymph nodes, and parts of the stomach and upper half of the small intestine (duodenum), the gallbladder and common bile duct (687). Chemotherapy using gemcitabine is performed after the procedure along with radiation therapy to prevent disease relapse (688). Techniques used to diagnose pancreatic cancer include endoscopic ultrasound (EUS), computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic retrograde cholangiopancreatography (ERCP) along with cytological and histological methods (686-690). Early stage pancreatic cancer is commonly asymptomatic and are hard to distinguish those from pancreatic cancer versus pancreatitis (686-690). Currently there are putative biomarkers that are being used for detection of early stage pancreatic cancer, which include carbohydrate antigen 19-9 (CA 19-9) (690,691). However, the American Society of clinical Oncology does not recommend such screening due to lack of specificity, false positives/negatives, and lack of consistency (692). The conspicuous paucity of effective biomarkers for pancreatic cancer truly accentuates the urgent need for elucidating the molecular mechanism of pancreatic cancer formation and how it evolves from early to late stage disease.

Pancreatic cancer: lifestyle and diet

Signs and symptoms of pancreatic cancer include abdominal and back pain, unexplained weight loss, lack of appetite, and light colored stools (685,693). There is an age factor associated with pancreatic cancer as those below the age

of 40 rarely contrive the disease and more than half occur in individuals over the age of 70 (685,693). Risk factors include diabetes, obesity, smoking, and sedentary lifestyle (693,694). Smoking is an established risk factor for pancreatic cancer, and the International Pancreatic Cancer Consortium conducted a nested case control study looking at smoking intensity, duration, and cumulative smoking dose (694,695). The results clearly showed that smokers had a greater risk than non-smokers did. It should also be noted that risk of pancreatic cancer was reduced to levels of that of a never smoker 20 years after quitting (694,695). Obesity and a diet consuming highly processed red meat is also directly associated with higher pancreatic cancer risk. These meats contain high amounts of nitrite, which can form nitrosamines, which are known carcinogens (658-660,696). Epidemiological studies show a strong correlation between increased pancreatic cancer and consumption of meat cooked at high temperatures (690,691). Not only can diet have deleterious effects on cancer but it can also have a strong preventive effect. Indeed, diets rich in fruits and cruciferous vegetables have shown to not only reduce the risk of pancreatic cancer, but also colorectal and other forms of cancer (25-27,658-660,690,691). Pancreatitis and diabetes are potential risk factors, with pancreatitis also being common in heavy drinkers and there is an increased risk four fold for pancreatic cancer in individuals with pancreatitis (692,693). Approximately 25% of pancreatic cancer patients have diabetes mellitus and more than 40% present with hyperglycemia and other pre-diabetes presentations (692,693). Diabetes Type-II is also implicated in

pancreatic cancer with 50% increased risk however, if there is a direct link it has not yet been unequivocally determined.

Pancreatic cancer: hereditary and genetic component

There is also a hereditary component associated with pancreatic cancer. Hereditary syndromes due to mutations in tumor suppressor genes are also risk factors but this accounts for approximately 5% of cases (694). Familial atypical multiple mole melanoma syndrome (FAMMM), hereditary pancreatitis (PRSS mutations), Lynch syndrome (MLH1, MSH2 and other MMR enzymes) Peutz-Jehgers syndrome (LKB1), Cowden syndrome (PTEN), germline mutations in BRCA2 and p16 mutations represent common examples of these (418-422,694-698). Mutations are linked to PanIN pathogenesis model. KRAS mutations are observed in approximately 80% of pancreatic cancer; other mutations include ErbB2/HER2, CDKN2A/p16, TP53, and DPC4/SMAD4 (699-702). Early PanIN lesions exhibit KRAS mutations that promote sustained proliferation along with overexpression of HER2, which is rarely expressed in normal pancreatic ductal epithelium but overexpressed in approximately 92% of pancreatic ductal dysplasia (699,700). Activation mutations can also present without atypia but frequency and prevalence of KRAS mutations increases with atypia in PanIN lesions (685,868,699,700). Surprisingly HER2 expression is absent in poorly differentiated invasive carcinomas which suggests that this gene is involved in early stage growth and survival but is repressed or lost in stages of EMT and invasion/metastasis (700,703). HER2 also activates Ras through the

Raf/MEK/ERK pathway to synergistically augment cell proliferation and growth mechanisms in early stages of pancreatic cancer (699,703).

Many tumor suppressors follow the two-hit hypothesis model and require biallelic mutations and this seems to be a driver in metastatic/advance pancreatic tumor development. The tumor suppressor gene *CDKN2A* encodes two potent cyclin dependent kinase inhibitors, p16 and p14ARF (206-209). The protein p16 inhibits entry of cells into S phase from G1 phase by binding to and inhibiting Cdk4/Cdk6, preventing them from associating with cyclin D1 (206-209). This prevents the hyperphosphorylation of Rb, which enables Rb to suppress cyclin E and A transcriptional activator E2F (188,206-209). The p16-Rb axis is inactivated in 95% of invasive pancreatic carcinomas and *CDKN2A/*p16 expression is progressively lost in 30, 55, and 75% of PanIN-1, PanIN-2, and PanIN-3 lesions respectively (704,705). Later stage carcinomas show deletions in tumor suppressor genes *TP53, DPC4,* and BRCA2 (374-376,594-596,704,705). The absence of p53 is observed predominantly in PanIN-3 and 75% of invasive pancreatic carcinomas (704,705). DPC4 stands for “deleted in pancreatic cancer locus 4” and is absent in 30 to 40% of PanIN-3 lesions and 55% of pancreatic carcinomas (704,705). It encodes the protein Smad 4 which is important in TGF-β signaling pathway (374-376). Upon activation of TGFβRII/I, Smad 2 and 3 are phosphorylated and this enables binding of Smad 4, which translocates to the nucleus and transcribes growth-inhibiting genes (374-376). BRCA2 (along with BRCA1) are tumor suppressor genes (described in the breast cancer section) and

is mutated in only PanIN-3 lesions and less than 10% of invasive pancreatic carcinomas (594-596,704). Diagnostic screenings for pancreatic cancer may be focused on individuals with specific genetic backgrounds, however, there is no single “smoking gun” gene that can be used to detect early pancreatic cancer.

Specificity Protein (Sp) Transcription Factors

Specificity proteins in embryological and normal biology

Specificity proteins (Sp proteins) such as Sp1 are members of a family of transcription factors which have similar structures to transcription factors known as Krüppel-like factors and the Sp/KLF family has 25 members (706-709). KLF family members share strong homology with a segment polarity gene called Krüppel, which is an important maternally derived factor in the development of the fruit fly syncytium (*Drosophila melanogaster*) (706-709). There are 9 Sp factors which contain three tandem Cys_2-His_2 (C_2H_2) type zinc fingers, which confer DNA binding capabilities (709,710). Sp proteins preferentially bind to GC rich regions with the consensus sequence [5’-GGGGCGGGG-3’] (Figure 50) as opposed to the KLFs, which bind CACCC boxes and the sequence [5’-GGTGTGGGG-3’] (708-710). Sp transcription factors are divided into two groups with Sp1-4 having very similar domain structure and organization whereas 5-9 have domain structure that more closely resemble KLFs and seem to be truncated forms of Sp 1-4 lacking the N-terminal TADs (706-710). Sp1-4 are characterized by an N-

terminal transactivation domain that contains glutamine rich regions proximal to serine and threonine rich regions (Figure 51) (708,710,711). These constitute transactivation domains A and B respectively, which bind with other transcription factors and enhance transactivation of target genes. Sp transcription factors also contain a zinc finger DNA binding domain (D), with the exception of Sp3 which has an extra inhibitory domain and Sp2 only having one transactivation domain (708,710,711). Sp2 also contains a histidine to leucine mutation in the first zinc finger which results in a switch to a GT binding preference for Sp2, whereas Sp1,3,4 zinc fingers are conserved (712,713). It has been shown that Sp1,3,4 share similar functional roles and have the highest physiological relevance when it comes to cancer (to be discussed) (706-710,714-718]). Although the

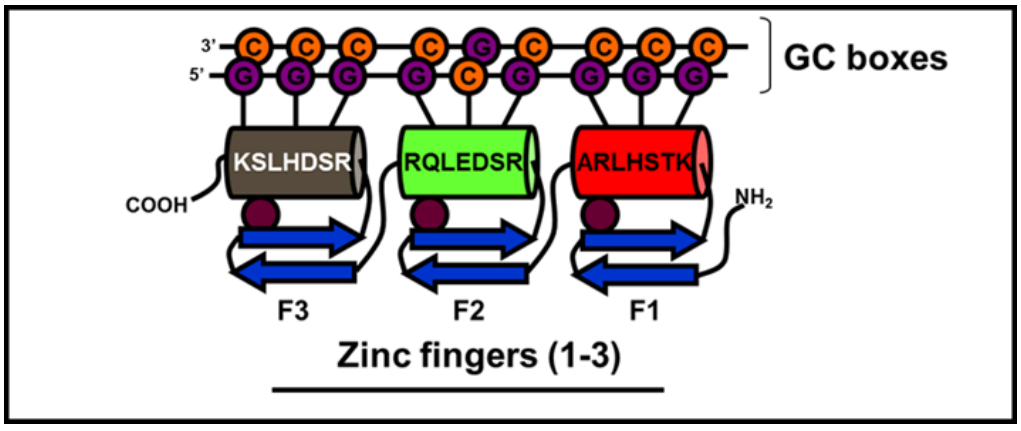

Figure 50: Sp proteins bind GC boxes. Sp proteins 1,3 and 4 bind to GC rich regions by using Cys2His2 type Zinc Fingers.

Sp transcription factor transactivation domains are similar in terms of their overall amino acid content, there actual primary structures differ and this is one reason why different Sp proteins can bind to different partners and have differing functions and physiological responses (711,712). There is also the C-domain, which is a highly charged region that is C-terminal with respect to the transactivation domains and promotes binding of the zinc fingers to the DNA (708,709,711,712). The first zinc finger has the highest affinity and sequence specificity and the zinc fingers seem to play a role in nuclear localization as deletion of any of the zinc fingers not only abolished DNA binding but abrogates nuclear localization (711-713). In contrast to other Sp members, Sp1 contains a multimerization domain at the further C-terminal region that mediates super-activation of promoters containing multiple adjacent Sp1 sites, as well as a Btd domain (buttonhead, the protein that is the putative fruit fly homolog to Sp1) (706,708,711). Sp1 molecules interact and form oligomers that promote associate with multiple proximal promoter sites with distal enhancers that loop DNA, that expose Sp1 for further protein-protein interactions and post-translational modifications (709-711).

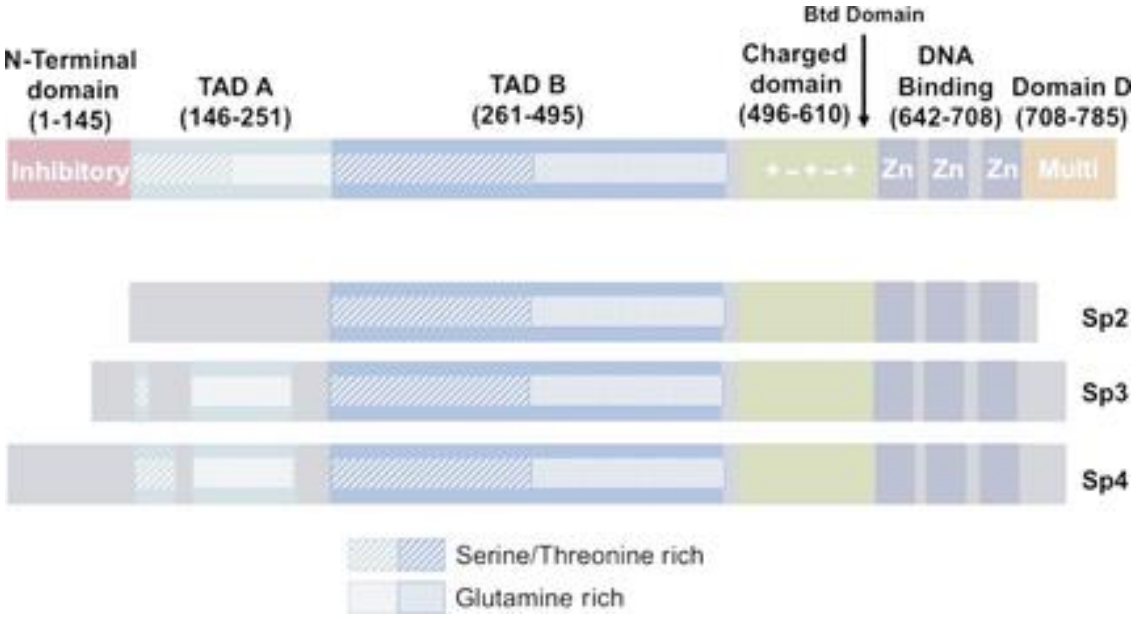

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Figure 51: Sp protein transcription factors 1-4. Sp proteins are Cys2His2 zinc finger DNA binding transcription factors. They share a high level of homology and also have significant differnces. They have two transactivational domains (TAD) A and B which correspond to glutamine rich and serine rich regions respectively. Sp1 is unique in the fact it contains an N-terminal inhibitory domain, and a C-terminal multimerization domain.

The Sp family genes are located adjacent to a homeobox (HOX) gene cluster; which encode genes that dictate the anterior-posterior axis of the embryo (719). The Sp transcription factors are important for embryological development and Sp1 knockout mice die after 11th day of gestation with mice observing multiple abnormalities beforehand (171,720). Sp3 mice display impaired ossification, tooth development, and die shortly after birth succumbing to respiratory failure (721). Sp4 knockout mice exhibit embryonic lethality; however, about 33% of the mice survive with severe growth retardation and male infertility (722). This

underscores the physiological important of the Sp proteins during development. Sp proteins directly bind with basal transcriptional machinery, including the TATA binding protein associated factors (TAFs) and other cofactors, which include many other basal transcription factors, including histone acetyl transferase, HDACs, histone methyl transferases etc (383-385,706,709,723-726). Indeed, many mammalian genes are regulated by Sp proteins, which play a critical role in carcinogenesis by being important effectors of cell proliferation, growth, angiogenesis, and migration/invasion (712-716,727,728).

Specificity protein 1 (Sp1)

Sp1 full-length protein is highly conserved among mammalian species and they are defined by their DNA-bonding domain (708,710,711). Sp1 is known to have three isoforms (A, B, and C) which are alternate splice variants, with B using an alternative start codon and isoform B being an alternate splice variant of exon 3 (lacking amino acids 55-102) (708,710,711). The role that these isoforms play in development, as well as cancer, has not been well characterized however, and their roles could be functionally redundant or compensatory (710,711). The Sp1/KLF factors play multiple roles in development and their expression and interplay is regulated during development. The DNA binding zinc finger domain exhibits the highest degree of conservation and is conserved in fish birds, reptiles, and even fruit flies (there has yet to be one in *saccharomyces cerevisiae* or *pombe*). The protein buttonhead in *Drosophila melanogaster* is the fruit fly homology of Sp1 and there is a small region of homology in the Sp1 protein that

is the eponymous buttonhead domain (706-710,728,729). Sp1 transactivation domains exhibit no sequence specificity among species and sequence specificity is conferred by the Sp1 DNA binding domain, which also demonstrates the only structural element of the protein (706-710). Domains A, B, C are largely unstructured and this is believed to confer the high degree of interaction with a large number of binding partners, and the ability of the Sp proteins to regulate a myriad of genes (706-710,726-729). Along with basal transcription factors Sp1 is known to interact with p53, E2F, c-Myc (genes involved in the cell cycle), NF-YA (a p53 regulated gene that induces apoptosis) SWI/SNF complex, HMGA1 and 2, DNMT1, HDAC1 and p300 (chromatin modifying complexes) (706-710,726-731)

Post translational modifications of Sp1

Many of the post-translation modifications of Sp1 have unknown consequence, however many have been documented and play a role in protein stability, DNA binding, transcriptional activity, and subcellular localization. For example, phosphorylation by Erk1/2 or JNK1 on T355 leads to enhanced transcriptional activity of Sp1 resulting in induction of apolipoprotein A-1 which is mediated by enhanced EGFR expression (732), whereas atThr278/739 by JNK1 leads to decreased stability and transcriptional activity (733). T287 phosphorylation has a similar effect and is important in the cell cycle by enhancing Sp1 stability (734). Similar stability and DNA binding effects are observed by CDK2 during the cell cycle by its phosphorylation of S59 on Sp1 (735). Phosphorylation of Sp1 can also lead to its degradation as evidenced by the

phosphorylation mediated by GSKβ on S728 and S732 (736). MAPK dependent phosphorylation can also upregulate Sp1-mediated transcription of VEGF and gastrin (317, 737). Glycosylation seems to play an important role in nuclear localization of Sp1 and O-linked Glycosylation of S612, 641, 698, 702 and T640 (as well as phosphorylation by DNA polymerase ζ on S612, 641 670 and T640, 651, 668, result in nuclear accumulation of Sp1 and activation of calmodulin (738-747). Whereas dephosphorylation of Sp1 by PP2A (T681) leads to increased chromatin association and increased cell proliferation in primary human T-lymphocytes (748). Sp1 acetylation (K703) is associated with loss of binding to gene promoters involved in apoptosis and cell cycle arrest in colon cells and deacetylation of Sp1 (K703) recruits p300 HAT to the promoter of 12(S)-lipoxygenase gene and upregulates its expression (749). The posttranslational modifications of Sp3 and four are not well understood; however, it is likely they are regulated as described for Sp1. Indeed, the degree of post-translational modification, both in number and type, emphasize the vast and diverse roles Sp proteins plays in cancer cell function and physiology.

Sp3 and Sp4

Sp3 has the highest degree of homology to Sp1 and they can act in concert or Sp3 can exhibit repressive activity on some genes (706-710,750,751). Sp3 has an extra inhibitory domain that is located just N terminal to its DNA binding domain and C-terminal with respect to the Transactivation domains and this domain mediates cell context-specific transcriptional repressor activity (706-710,749-

751). The D domain of Sp3 differs from that of Sp1 and this confers propensity to serve as an inhibitory factor of Sp1 transcriptional activity (750,751). Sp3 also lacks the multimerization domain of Sp1 and cannot induce super activation of genes as observed for Sp1 (750,751). Sp1 and Sp3 have equal binding affinities for GC rich boxes; therefore, their stoichiometric ratios, proximity to cognate promoters, subcellular location, and posttranslational modification status play a critical role in how they regulate genes. Sp1 binding also does not display cooperative binding since there is an equal likelihood of either Sp1 or Sp3 is observed after a single molecule of Sp1 binds to a GC-rich promoter (706,708,712,750,751). For genes that require multiple Sp1 molecules to bind on a promoter to enhance activation Sp3 can serve as a potent inhibitor of such genes (706,708,712,750,751). A fundamental example of this phenomenon can be observed in the regulation of the human topoisomerase IIa promoter (752). The activation of topoisomerase IIa promoter begins by Sp1 molecules binding to proximal promoters and then to distal enhancers (752). The loop is made and the Sp1 molecules, therefore enabling super activation of the topoisomerase IIa gene, generate a bridge (752). Sp3 competitive binding for either the proximal or distal promoter site leads to repression of the topoisomerase IIA gene (752).

Sp4 is similar to Sp1 and Sp3, however it has properties that make it distinct and its functions are not redundant (753-756). Sp4 serves as a transcription factor that has a highly homologus DNA binding region to that of Sp1, however, unlike Sp1, it cannot participate in synergistic cooperative activation

through adjacent binding sites (706,708,710,712,750, 753-756). Sp4 –mediated transactivation can also be suppressed by Sp3 and deletion of Sp4 results in phenotypically normal mice until they are born (724). Many die after 4 weeks but the ones that survive exhibit growth retardation, delayed sexual maturation, inability to breed, and underdeveloped thymus, spleen and uterus in females (724). One example of the unique transcriptional role of Sp4 is in the transcription of the beta-subunit in the cyclic guanosine monophosphate (cGMP)-phosphodiesterase, which is critical in phototransduction in the rod photoreceptors and as a co-transactivator with cone-rod-homeobox (Crx) on the promoter of rod opsin (757). Sp4 and Sp3 are indispensable for the murine gamma-aminobutyric acid type A receptors (GABAA-R), which is vital for the fast inhibitory synaptic transmission in neurons (758). Sp4 also plays a role in cerebral maturation and controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning and regulates the transcription of AMPA receptor subunit GluA2 (Gria2) (759). Less is known about Sp4 but emerging evidence is demonstrating its nonredundant and plays a unique role in cancer (712-716,760,761). In melanoma cells, Sp4, along with c-Myc and HIF-1α, cooperate in the activation of arginosuccinate synthetase (AS), which confers resistance in these cells to arginine deiminase treatment (762). We have also demonstrated that Sp4 is critical for inhibiting apoptosis in colon cancer cell lines such as SW480, breast cancer cell line SKBR3, and lung cancer cell line A549 (763-765). Integrins such as β1-integrin and α5-integrin are regulated by

Sp4 in RKO colon cancer cell line and pancreatic cancer (L3.6pL, MiaPaCa2, and Panc1) cell lines and not by Sp1. Studies in this laboratory have also uniquely demonstrated that Sp4 is a direct co-activator of the PAX3-FOXO fusion gene product in ARMS. This highlights the emerging nonredundant role of Sp4 in cancer and that its potential value as a drug target.

Sp proteins in cancer

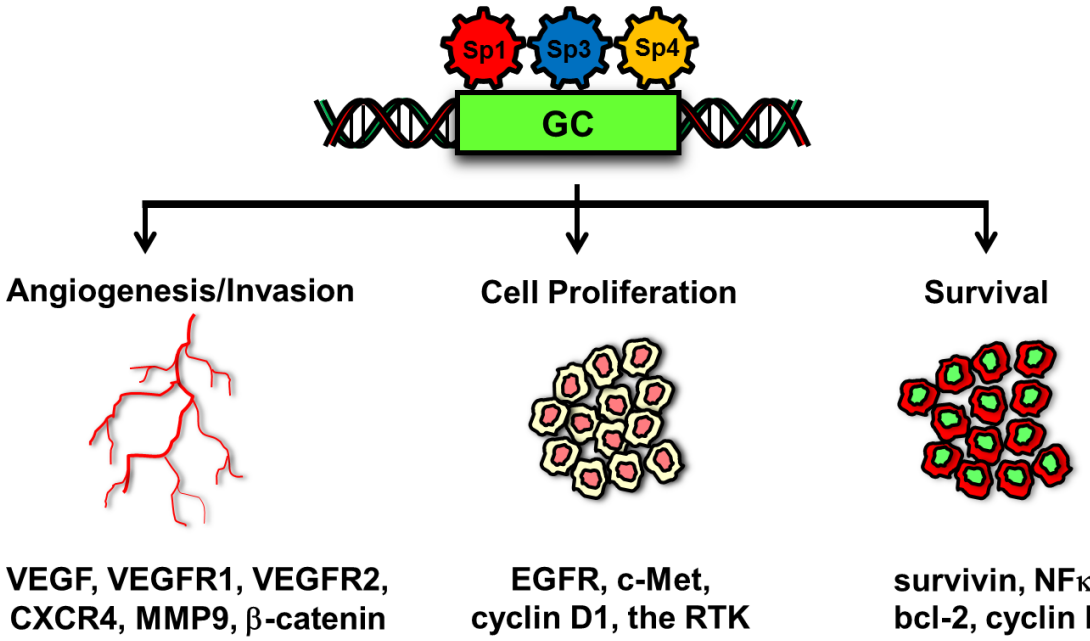

Figure 52: Sp proteins in cancer. Sp proteins recognize GC rich regions within promoters of oncogenes. This involves monomeric or oligomeric homo or heterodimeric Sp protein complexes which bind to the promoters of genes involved in angiogenesis (VEGF, VEGR1, VEGFR2, CXCR4, MMP9), cell proliferation (EGFR, c-Met, CD1, IGF1R) and survival (bcl-2, survivn).

The role of Sp transcription factors in cancer has been reported in multiple cancer types including pancreatic, colon, bladder, GBM, prostate, lung, and breast cancers (712-716,765-772). We have described and characterized these genes

as non-oncogene addiction (NOA) genes, which is distinctively different from the canonical distinction of a bonafide oncogene addiction in cancer (77). Oncogene addiction refers to a gene (either amplified, overexpressed, or a mutant constitutively active form is endogenously or ectopically expressed such as through genetic translocation or via transposon activity) that a cancer cell depends on not only for its transformed phenotype but also its survival. Examples of this include HER2/ErbB2 overexpression in breast cancer, K-ras in colon and pancreatic cancer, bcr-Abl fusion gene product in CML, and EGFR in NSCLC. These genes are vital to normal tissue function and are expressed at moderate levels. However, Sp proteins have minimal expression in adult tissues and their expression decreases with age but Sp1, Sp3, and Sp4 are highly expressed in most tumors (710-716,766-772). This is a distinguishing characteristic of a NOA and Sp proteins represent a novel and promising class of NOAs that are not only vital to tumor growth and survival but can be targeted by drugs. Sp1 overexpression is observed in colorectal, gastric, breast, thyroid, and brain tumors as compared to their normal tissues counterparts and its expression has been demonstrated to be a negative prognostic factor for these cancers (710-716). The prognostic relevance of Sp3 and Sp4 have not been reported even though their expression levels follow the same pattern as those observed with Sp1 (710-716,766-772). Sp proteins have been demonstrated to be master regulators of genes essential for cancer cell morphology, physiology, proliferation, growth, and migration/invasion (766-772). Sp transcription factors bind to GC-rich promoters

and induce expression of a myriad of pro-oncogenic factors that include survivin, XIAP, EGFR, VEGF, VEGFR2, EGFR, IGF1r, c-MET, cyclin-D1, PDGFR, bcl2, c-fos, c-jun, E2F1, TGF-α, and TGFβRII (Figure 52) (710-716, 763-772). Thus drugs that downregulate expression of Sp transcription factors would also down-regulate multiple Sp-regulated pro-oncogenic factors and therefore be highly-effective anti-tumor agents.

Sp proteins as drug targets

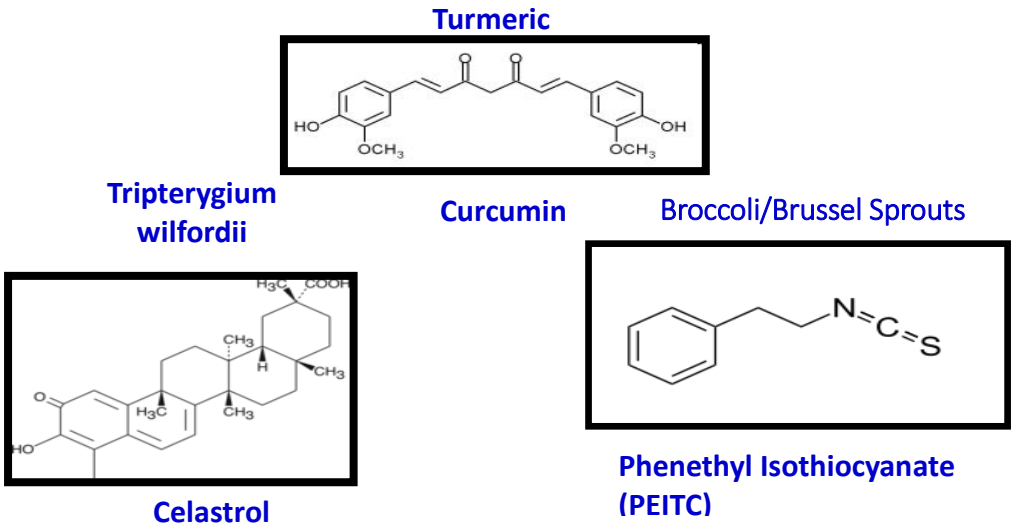

Figure 53: Sp proteins as drug targets for cancer. Sp proteins are downregulated in both an ROS -dependent (celastrol, curcumin, PEITC, betulinic acid, HDACi) ROS-independent (tolfenamic acid), and proteasome dependent (metformin, betulinic acid). They are composed of triterpenoids (betulinic acid, celastrol), isothiocynates (PEITC, BITC), NSAIDs (sulindac sulfide, tolfenamic acid). The drastic differential in expression of Sp proteins in cancerous vs. normal tissue and the fact that they compound exhibit low toxicity in normal tissue demonstrate the chemotherapeutic potential of Sp targeting drugs.

Several strategies to decrease Sp1, Sp3, and Sp4 in cancer cells and tumors have been reported (706-710,712-716,766-772). These include enzyme/chemical dependent modulations, drugs that competitively inhibit their

DNA binding or abrogate GC-rich DNA binding regions (i.e. mithramycin), GC-rich oligonucleotides that squelch Sp proteins and prevent them from binding to promoters, NSAIDS (i.e. tolfenamic acid, sulindac sulfide), and drugs/chemicals that induce ROS and downregulate Sp proteins (i.e. AsO_3, celastrol, curcumin) as outlined in Figure 53 (641-648, 766-772). Studies in this laboratory have shown that the cannabinoid, 2,3-dihydro-5-methyl-3-([morpholinyl]methyl)pyrollo(1,2,3-de)-1,4-benzoxazinyl]-[1-naphthaleny]methanone [WIN 55,212-2, (WIN)], represses Sp proteins in a protein phosphatase 2A (PP2A) dependent manner by disrupting the miR-27a-ZBTB10 interaction in glioblastoma multiforme (GBM) and colon cancer cells (773). The pentacyclic triterpenoid betulinic acid also downregulates Sp proteins via binding cannabinoid receptors in a proteasome-independent (mir-27a/ZBTB10) and dependent manner which target YY1 and HER2 (645,774). The biguanide antidiabetic drug metformin inhibits Sp protein expression by activating MAP kinase phosphatases (MKP) MKP1 and MKP5 which lead to proteasome-dependent degradation of Sp proteins (775).

Sp proteins as targets of ROS inducing agents by targeting c-Myc

ROS- inducing agents are highly effective at down regulating Sp proteins. These ROS inducing agents decrease Sp protein expression by a pathway elucidated, namely the c-Myc-miR17-92-ZBTB-Sp pathway characterized in our laboratory (641-648,768,769). C-Myc is an oncogene and is overexpressed in approximately 20% of all cancers and is the cellular homolog (hence the c in c-Myc) of v-Myc which is essential for avian retrovirus survival and viral induced

carcinogenesis (776-778). There are four other isoforms of Myc, which include N-Myc, L-Myc, S-Myc, and B-Myc (776-778). C-Myc has three conserved regions in its N-terminal called Myc boxes (MB) designated MB-MB-II, MB-III, and I (776-778). These regions are found in Myc family members and are critical for protein-protein interaction with many proteins, which regulate its expression, activity, and localization (776-778). C-Myc enhances transcription by recruiting transcription factors such as P-TEFb (positive transcription elongation factor b) (779). These MB regions also recruit kinases, which regulate its activity and degradation (779). Three phosphorylation sites within MB-1: T58, S62, and S71, are regulated by kinases GSK3α and β, MAP kinase ERK 1/2 and CDK kinase, and Rho associated kinase (ROCK) respectively (779,780). The latter two phosphorylation sites enhance stability and activity of c-Myc whereas T58 phosphorylation promotes association of Skp2 E2 ubiquitin conjugating enzymes with its E3 ligase counterpart FBW7 to induce polyubiquitination and proteasome-dependent degradation (779,780). The S62 site however is critical for docking for both GSK3β and the ubiquitin ligase complex as demonstrated by mutational analysis (776-780). These MB domains also recruit chromatin-modifying complexes, which includes TIP48/49 and TIP60, which are a histone acetyltransferase (HAT) complex. Other proteins called TTRAP and p400, an adaptor protein and histone acetyltransferase exchange factor for p300, recruit HATs such as GCN5 and p300 respectively (776-780). The C terminal domain (CTD) contains a bHLH (basic Helix-Loop-Helix) motif critical for DNA binding, and a leucine zipper, which

mediates interaction with transcriptional activator binding partners like Max, Miz1, and p300 (776-780). There is also a NLS (nuclear localization signal) just N-terminal with respect to the CTD (776-780). Expression of c-Myc is mainly controlled by the Nuclease hypersensitivity element III-1 (NHEIII-1) which when bound by transcriptional activators enhances expression. Negative regulation is conferred by 5'-UTR cis elements called negative regulatory elements (NRE) 1 and 2 which bind to proteins such as CTF (776-780). 5' regions of the c-Myc gene also contain poly-purine tracts, which can create guanine-rich duplexes, and quadraplexes that repress transcription (776-780).

The c-Myc/miR17-92/ZBTB/Sp pathway

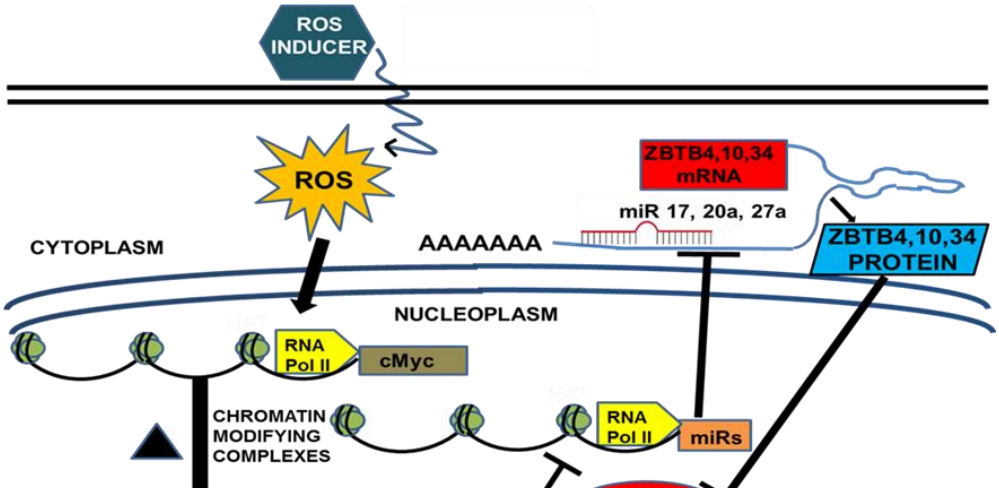

Figure 54: The c-Myc/miR17-92/ZBTB/Sp pathway. This pathway has been well characterized by our lab and involves an ROS inducing agent which promotes shifts within the chromatin modifying complexes and represses transcription of the cMyc gene. cMyc binds to E-boxes within the promoters of miR-17, miR-20a, and mR-27a. In cancer cells these miRs bind to the 3 UTR of ZBTB 4, 10, and 34 mRNA and suppress their translation. As result of the ROS inducing agent there is an upregulation of ZBTB protein expression. The proteins are Zn2+ finger BTB binding proteins which compete with Sp proteins for GC rich regions (presumably they concommitently suppress Sp expression because Sp proteins contain GC-rich regions within their promoters. This leads to a decrease in cell groth, migration, and induction of apoptosis.

The pathway discovered in our laboratory involves ROS-dependent epigenetic silencing of c-Myc (Figure 54) (641-648,766,768,769). Induction of ROS leads to chromatin modifying complexes (i.e. PRC1, PRC2, NCOR, SIRT1) from non-GC to GC-rich regions in a thus far an unexplained mechanism (781,782). Although a substantial amount of c-Myc transcriptional regulation is controlled by an upstream cis-element known as NHEIII, there are also GC rich regions in the c-Myc promoter and this leads to ROS-dependent epigenetic silencing of c-Myc (648,766,768,769). C-Myc regulates expression of miR 17-92 cluster (as well as miR27a) by binding to E-Box recognition motifs (CANNNTG) (641-648,766,768,769).

Thus downregulation of Myc results in a rapid decrease in the expression of miR-17, miR-20a, and miR-27a, which are negative regulators of zinc finger and BTB domain containing DNA binding proteins, namely the “Sp” transcriptional repressors ZBTB 4, ZBTB 10, and ZBTB 34 that compete with Sp proteins for binding to GC rich sites (641-648,766,768,769). The miRs bind to the 3’ UTR of the ZBTB mRNA sand repress their expression; therefore, the decrease in the miR expression, increases in expression of ZBTB 4, 10 and 34 expression, and the subsequent inhibition of Sp proteins and pro-oncogenic Sp-regulated genes (641-648,766,768,769). In this thesis, I will demonstrate the role of Sp proteins in multiple cancer types and how these Sp proteins can be targeted through an ROS dependent mechanism that is similar to the pathway outlined in figure 53, using hydroxamic class HDAC inhibitors panobinostat (LBH589) and vorinostat (SAHA

or suberyolanilide hydroxamic acid. Antipsychotic drugs such as penfluridol also induce ROS and suppress triple negative breast cancer (TNBC). Penfluridol also decreased expression of integrins α6 and β4 and our studies now show that penfluridol downregulates multiple integrins via induction of ROS and the pathway illustrated in figure 54.

NR4A Nuclear Receptor: NR4A1

Nuclear receptors

Nuclear receptors comprise of a large family of intracellular transcription factors that are involved in diverse developmental and physiological processes such as organogenesis, homeostasis, metabolism, steroidogenesis, reproduction, and neurological health (558,563,783). There are 48 human nuclear receptors, which can be grouped into four general categories: Type I, Type II, Type III, and Type IV (Figure 55) (558,563,783). Type I nuclear receptors consists of receptors associated with steroid-induced pathways such as the androgen receptor (AR) and the estrogen receptors (ERα and ERβ), progesterone receptor (PR) as well as the glucocorticoid receptor (GR) (558,563,783). These receptors exist in an inactive state in the cytosol associated with heat shock (HSP) proteins, which prevent premature dimerization, and unfolding of the receptor (558,563,783).

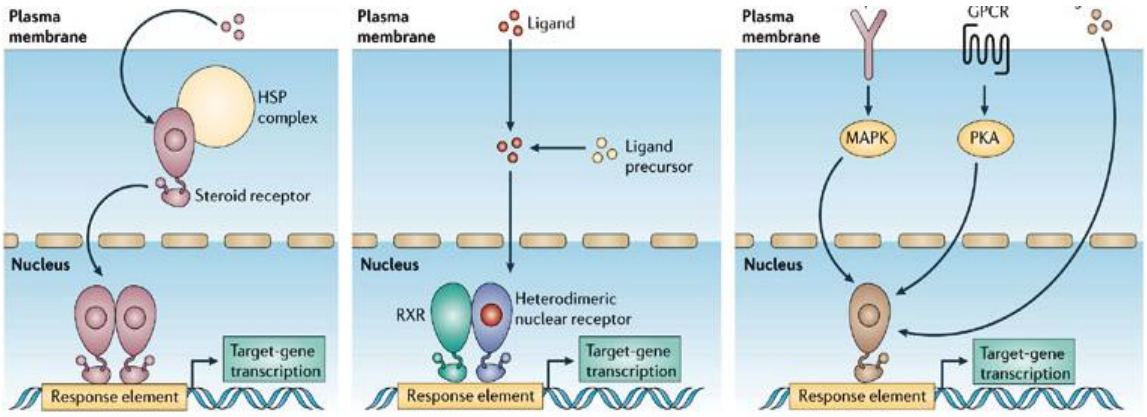

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Figure 55: The nuclear receptor superfamily. Nuclear receptors are a very large and diverse family which play extensive roles in development, reproductive biology, normal physiology, metabolism, and cancer progression. Group I receptors exist complexed in the cytoplasm with Hsp90 molecules and upon ligand activation translocate to the nucleus as homodimers and bind to inerted repeat response elements (ER, AR, PR, GR). Group II receptors reside in the nucleus regardless of ligand binding and heterodimerize with the RXR (retinoid X receptor), binding to direct repeat response elements. These include The vitamin D receptor (VDR) and pregnane X receptor (PXR). Group III receptors upon ligand binding can exist in cytoplasm or nucleus but bind to direct repeats as monomers (sometimes dimers) (steroidgenic factor 1 (SF-1)). Group IV exist as either monomers or dimers but bind to only half site HRE (hormone response elements)

Upon ligand binding to the receptor, the HSP proteins dissociate, and the receptor translocates to the nucleus to form homo-dimers, that bind sequences known as hormone responses elements, which consist of two inverted half sites, which are separated by a variable length of DNA (558,563,783). This confers specificity for the hormone receptors, for example, an estrogen response element contains AGGTCA-nnn- TGACCT, with n meaning any nucleotide (783,784). Type II receptors are located in the nucleus and they heterodimerize with the receptor RXR (retinoid X receptor) (558,563,783,784). Ligand binding induces recruitment of cofactors/coactivators resulting in activation of target gene expression; they stimulate expression of genes that contain direct tandem half

sites that are spaced by a variable length of DNA (558,563,783,784). Examples of these receptors are the RAR (retinoic acid receptor) and the PXR (pregnane X receptor) (558,563,783,784). The PXR is a very important receptor, which along with binding pregnane related compounds binds to drugs such as rifampicin and ketoconazole (783,785). PXR is an important drug metabolizing enzyme that stimulates expression of cytochromep450 3A4 (CYP3A4) which is a very important drug metabolizing enzymes that metabolize drugs such as rifampicin (785).

Type III nuclear receptors, are similar to Type one in that they bind as homodimers, however they recognize and bind to direct as opposed to inverted repeats (558,563,783-785). SF-1 (steroidogenic factor 1) is a Type III receptor that is involved in puberty and sexual development (also known as the NR5A1) (786). It upregulates the expression of genes such as CYP11A1 (cleaves the side chain of cholesterol in the steroidogenesis pathway), steroid acute regulatory protein, Sox9, and genes critical in the hypothalamic-pituitary gonadal axis (786,787). Type IV receptors most often bind as monomers, but can dimerize to form either in a homo- or hetero-dimers (563,783,784,786). There are seven subfamilies based on sequence homology which include the thyroid hormone receptor-like (NR1), the retinoid X receptor -like (NR2), the estrogen receptor-like (NR3), nerve growth factor IB-like (NR4), steroidogenic factor-like (NR5), germ cell nuclear factor-like (NR6) and miscellaneous (NR0) receptors (786-788).

Orphan nuclear receptors: NR4A1

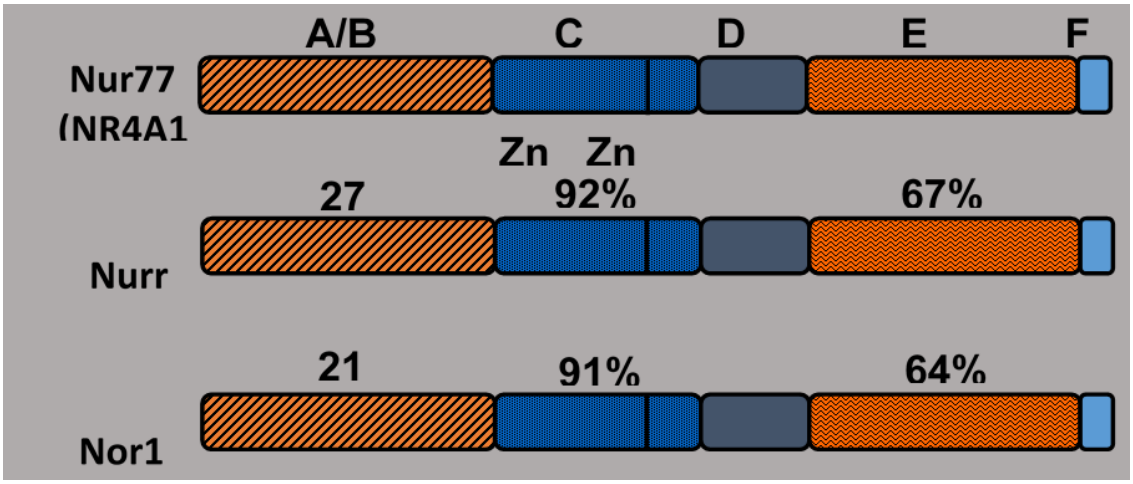

Figure 56: The nuclear receptor 4A (NR4A1) family. They Have high homology within their DNA-binding domains and have high variability within their AF regions (A/B and E/F). NR4A receptors are orphan nuclear receptors which have a vast role in neurological function, metabolism, steroidgenesis, and physiology. The NR4A1 receptor is overexpressed in the majority of solid tumors and has a major role as an oncogene. It serves as a transactivator of a myriad of genes involved in carcinogenesis. The other receptors NR4A2 and NR4A3 have lesser known roles in carcinogenesis and their exact contribution to cancer progression is being further investigated.

The orphan nuclear receptors, for which no known endogenous ligands have been identified, include COUP-TF (NR2F), TLX (NR2E), SHP (NR0B), PNR (NR2E3), GCNF (NR6A), and nerve growth factor IB-like subfamily (NR4A) (Figure 56) (786-789). The NR4A subfamily genes consist of three members that are subcategorized as group A (NR4A) and include NR4A1 [(nerve growth factor IB NGFIB), testicular receptor 3 (TR3), nurr77], NR4A2 [Nurr1 (nuclear receptor related 1)] and NR4A3 [Nor1 (neuron-derived orphan receptor 1)] (790-792). The first member, NR4A1 was initially identified as a nerve growth factor-induced early immediate gene in rat pheochromocytoma PC12 cells (790,793). NR4A receptors

exhibit a wide range of functions in humans (790,793-796). NR4A1 has been shown critical for metabolism in normal and neuronal cells and its aberrant expression has been implicated in Parkinson's disease (794). NR4A2 is important in neuron development and maintenance in the central nervous system and both NR4A1 and NR4A2 both orchestrate neuroendocrine regulation in the hypothalamic-pituitary adrenal axis (794,797-799). NR4A1 and NR4A3 are involved in glucose metabolism and modulate adipocyte differentiation, and NR4A3 is involved in skeletal muscle metabolism (790-792,795,800-802). There is also increasing evidence that NR4A family members play an important role in cancer (790-792, 803-809). Several studies show that NR4A receptors are involved in tumor and cancer cell growth, proliferation, apoptosis, DNA repair, and glucose and fatty acid metabolism (793-796, 800-802). The role of NR4A1 in cancer, however, has been extensively investigated and this thesis will provide evidence that NR4A1 regulates expression of pro-oncogenic factors and pathways.

NR4A1 in cancer

In many solid tumors NR4A1 is overexpressed and plays a role in cancer growth and physiology and is a negative prognostic factor for disease-free and metastasis-free survival (803-809). In leukemias and other hematological malignancies, the role of NR4A1 is equivocal and some studies have reported a tumor-suppressive like function in these malignancies (810,811). It has also shown that the subcellular localization of NR4A1 is also critical for determining the

function of NR4A1 (812-820). In the studies that demonstrate a tumor-suppressive role for NR4A1, these cells largely depend on its cytosolic and not its nuclear location (812-827).

In solid tumors, NR4A1 is primarily in the nucleus where it is constitutively expressed and serves as a transcriptional activator of oncogenes (790-793, 812-820). However, upon stimulation by some apoptosis inducers such as phorbol esters and retinoid-derived compounds, NR4A1 expression is induced and translocated to the mitochondria where it associates with bcl-2 to form a pro-apoptotic complex (812,813). It accomplishes this by exposing its BH3 domain (which as stated earlier confers pro-apoptotic activity) and inducing MOMP (812,813). This apoptotic pathway for NR4A1 has been reported in several types of cancer and a similar mechanism is observed in negative selection of T-cells (818). In colorectal cancer, NR4A1 indirectly activates Bax, which results in MOMP and cytochrome c release into the cytosol and induction of apoptosis (819). NR4A1 also suppresses colorectal tumorigenesis by inhibiting Wnt signaling by induction of β-catenin degradation (817). In gastric cancer cells, PKC signaling is activated and this promotes NR4A1 nuclear export (820).

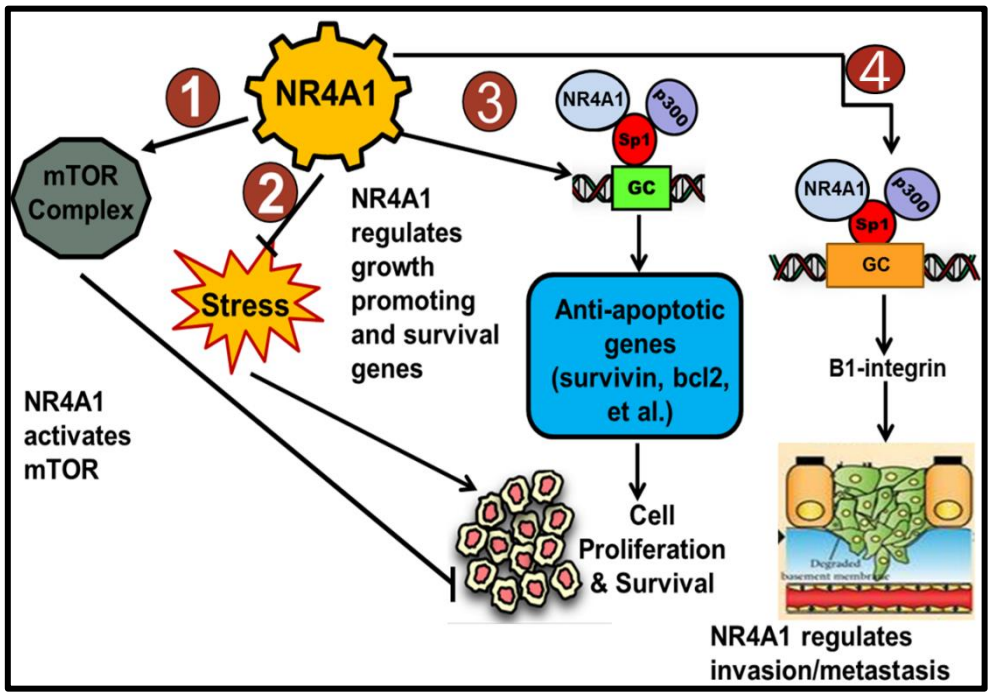

Figure 57: NR4A1 as an oncogene that is drug targetable. 1) NR4A1 regulates mTOR by binding to p53. P53 activates sestrin2 which activates AMPKα. 2) NR4A1 also regulates the expression of ROS modulators TXNDC5 (thiodoxin domain containing 5) and IDH1 (isocitrate dehydrogenase 1). 3)NR4A1 also forms a transactivational complex with Sp proteins and p300 histone acetyltransferase, which regulate expression of p regulated genes (such as bcl2, survivin, and EGFR). NR4A1 also participates in migration and invasion, which involves transcriptional upregulation of β1-integrin.

NR4A1 as an oncogene that is drug targetable

Our laboratory has focused predominantly on the nuclear functions of NR4A1 as a transcriptional activator of oncogenes and its role in promoting pro-oncogenic and pro-invasive functions (Figure 57) (803-809). We have discovered a series of C-DIMs (1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methane analogs) that bind NR4A1 and act as antagonists of this receptor. DIM-C-pPhOH (C-DIM8) is an NR4A1 ligand that acts as an antagonist that inhibits lung cell and tumor growth and this was due in part to inhibition of mTOR and decreased expression

of survivin being observed (803-812). The C-DIM/NR4A1 antagonist inactivated expression of survivin by disruption of an NR4A1/Sp1/p300 transactivational complex (803,804). This thesis will demonstrate the anticancer activity of C-DIM/NR4A1 antagonists in breast, kidney, colon, and pancreatic cancers by inactivating NR4A1-regulated oncogenes that are critical for tumor growth survival, and migration. Results of my studies will show that NR4A1 antagonists induce ROS by targeting key genes involved in maintaining levels of cellular reductants and increased ROS also induced sestrin2 (SESN2) which activates AMPKα resulting in inhibition of mTOR.

NR4A1 and nongenomic pathways: apoptosis and autophagy

NR4A1 also participates in nongenomic pathways that regulate apoptosis, survival, and migration pathways. NR4A1 binds with anti-apoptotic protein bcl2, which in normal conditions antagonizes Bax/Bak-induced apoptosis and prevents MOMP through homodimerization and heterodimerization with Bcl-XL (813,814). Certain compounds were demonstrated such as retinoid 3-Cl-AHPC (4-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid) and phorbol ester TPA (12-*O*-Tetradecanoylphorbol-13-acetate, an inducer of PKC signaling) to induce NR4A1 nuclear export through its NES (nuclear export sequence) by association with RXR (813-815). Through NR4A1 LBD, it binds to the N-terminal loop of Bcl-2, which displaces its BH4 (anti-apoptotic conferring domain) and exposes its proapoptotic BH3 domain that is concealed prior to this association event. This also reduced the anti-apoptotic propensity of Bcl-XL (813,814). NR4A1 can also

associate with other Bcl-2 family members such as Bcl-B and Bfl-1 (814). 3-Cl-AHPC is not unique in its ability to induce nuclear export of NR4A1 or association with bcl-2. Recently, it was reported that a coumarin derivative, apaensin, can activate p38 (MAPK14) kinase which promotes mitochondrial translocation of NR4A1 and its subsequent association with bcl-2 (816). In RAW264.7 T-cells, LPS can activate p38, induce phosphorylation of NR4A1 at T27 and T143, and induce apoptosis in these cells. NR4A1 also induces autophagy at the mitochondria by associating with Nix and ANT1 (adenine nucleotide translocator 1), with the latter being involved in MOMP and changes in mitochondrial inner membrane potential (817). Other alkaloid compounds such as dendrogenin A (DDA) induce NR4A1-mediated autophagy in cancer cells such as melanoma cancer cells (818). Induction of autophagy by NR4A1 is not always associated with apoptosis since certain activated receptors such as IGF1R and neurokinin-1 receptor (NK_1R) induce autophagy related to cancer cell survival, where NR4A1 is translocated to the cytosol with p53 and inhibits p53 in HEK293 cells (819). Nuclear export of NR4A1 induced by AHPN, an adamantine-derived retinoid similar to 3-Cl-AHPC, also induces nuclear export of NR4A1 and is dependent on c-jun N-terminal kinase (JNK) and AKT (820). NR4A1 can also localize to the ER, associate with TRAPγ (through LBD of NR4A1), and induce ER-mediated apoptosis (translocon associated protein subunit gamma) (821). This inhibits the ability of TRAPγ to bind calcium to the ER membrane and retain ER resident

proteins within the ER lumen, as well as the efflux of calcium that triggers apoptosis through SERCA (821).

NR4A1 nongenomic pathway: TGF-β

NR4A1 can also translocate to the cytosol and activate tumorigenesis and invasive/migratory pathways (822). It was reported that NR4A1 was necessary for TGF-β induced migration in TNBC cells (822). NR4A1 associates with RING ringer E3 ubiquitin ligases arkadia (RNF111) and RNF12, to promote the ubiquitination and subsequent proteasome-degradation of inhibitory Smad 7 and negative regulator of TGF-β (822). TGF-β is a tumor suppressor in early stage cancer but in advanced cancer TGF-β is a promoter of migration/invasion and EMT and in breast cancer NR4A1 is important for this response (822). In chapters V and IX of this thesis we describe the role and mechanism of action of NR4A1 in mediating TGF-β-induced TNBC invasion and the effects of C-DIM/NR4A1 antagonists as inhibitors of TGF-β-induced migration and EMT.

NR4A1 and β-catenin

β-catenin is a potent driver of TGFβ-induced EMT in cancer cell lines (52-54,823). The β-catenin protein contains 40 armadillo repeats (the *Drosophila melanogaster* homolog of β-catenin) (823,824). The repeats arrange into a rigid structure known as the armadillo domain (ARM) (823,824). This domain is found in many proteins that are important for cytoskeletal infrastructure (γ-catenin), development (APC β-catenin), and Wnt signaling (APC) (823-825). Along with

this central containing highly structured motif, β-catenin also contains two unstructured regions at both its N-terminus and C-terminus (823,824). With the N-terminus, there is a region for binding to an E3 ubiquitin ligase known as TrCP-1 (823,824). This promotes binding to the TRCP-1 protein, only when the N-terminal region is phosphorylated (namely by GSK3-β and CK-1). The C-terminal region of β-catenin contains a strong transactivator domain, with a structural helix that is just C-terminal to the ARM domain, which confers high transactivational propensity, termed HelixC (823,824,826). This region of β-catenin confers association with TCF/LEF transcription factors (namely LEF1, TCF1, TCF2, TCF3, and TCF4), which is important for the ability of β-catenin to act as a transcription factor as β-catenin itself cannot bind directly to DNA (826-828). TCF proteins bind to a conserved DNA binding sequence (A/T)(A/T)CAA(A/T)G in the minor groove within cognate promoters. One nucleotide within the LEF-1 consensus site (TTC**A**AAG) is indispensable for binding and abolition or mutation of this nucleotide abrogates LEF binding (827,828). These transcription factors are part of family of proteins known as high mobility group (HMG) proteins (826-828). These proteins are defined by the possession of a high mobility group (HMG) box that confers DNA binding propensity (826-828).

There is evidence that β-catenin and NR4A1 interact and regulate one another's stability (829-833) and one example shows an indirect correlation between the two proteins involving adrenal aldosterone-producing adenomas (APAs) Secondary hypertension is caused by primary aldosteronism (PA) (829).

PA is caused by APAs and it was recently demonstrated that β-catenin through the Wnt pathway promotes Angiotensin II-induced aldosterone secretion indirectly by activating AT1R, CYP21 and CYP11B2 by increased expression of NR4A1 (829).

The evidence for mutual regulation of NR4A1 and β-catenin is equivocal in a colorectal cancer. One study reports that CRC is exacerbated by colonic carcinogen deoxycholic acid (DCA) induced expression of NR4A1 and this mechanism involves β-catenin activating the transcription factor AP-1 (c-Fos/c-Jun), which binds the NR4A1 promoter and induces NR4A1 expression (830). Another study shows that NR4A1 and β-catenin coregulate one another and participate in a mutual feed-forward loop (831). β-catenin stimulated NR4A1 at the transcriptional level through HIF-1α and NR4A1 regulates β-catenin positively through a PI3K/Akt-dependent nongenomic pathway (831). However, other studies report that NR4A1 antagonizes expression and stability of β-catenin (832-834). For example, in Escherichia coli-induced peritonitis, NR4A1 downregulated transcription of β-catenin and the converse was observed in the lungs of NR4A1-deficient mice (832). Other evidence suggests that nongenomic NR4A1 antagonizes β-catenin expression by proteasome-dependent degradation of β-catenin *in vivo* and *in vitro* by treating cells with two digitalis-like compounds (DLCs), H-9 and ATE-i2-b4 (833). This proteasome-dependent degradation involves NR4A1 nuclear export and there was evidence that this export was dependent of the c-Jun N-terminal Kinase (c-JNK) (833). In hepatocellular

carcinoma (HCC), it was reported that NR4A1 participates in degradation of β-catenin and that NRDG1 (N-Myc downstream regulated gene) binds to NR4A1 and GSK-3β, to enable β-catenin to escape degradation (834). In chapter IX, we provide evidence demonstrating a role for β-catenin in NR4A1 regulation andNR4A1/TGFβ induced migration. We also show (chapter IX) that β-catenin binds directly to the NR4A1 promoter with TCF3, TCF4, and LEF-1. NR4A1 also contributes to the stability of β-catenin, since nuclear export of NR4A1 is required for β-catenin nuclear localization. Treatment with NR4A1 antagonists, or CRM1 (chromosomal maintenance 1 or Exportin 1(XPO1))-dependent nuclear export inhibitor leptomycin B promote cytosolic sequestration of β-catenin, which leads to a time dependent proteasome-dependent degradation of β-catenin. This dissertation will show that NR4A1 and β-catenin play a role in their mutual regulation, stability, and subcellular location. Previous reports and results obtained from studies in this laboratory significantly demonstrate that NR4A1 is a master regulator of pro-survival, invasive, and anti-apoptotic pathways and plays a critical nongenomic role in regulating TGFβ-induced migration and EMT in TNBC cells.

C-DIMs as novel NR4A1 ligands and antagonists

Studies from this laboratory show that a series of 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methane (C-DIM) analogs can bind NR4A1 and inhibit NR4A1-dependent oncogenic and invasive pathways (Figure 58) (803-809). The latter includes basal migration/invasion by NR4A1 serving as a transcription factor

for β1-integrin as well as a nongenomic and inducible migratory pathway stimulated by transforming growth factor β (TGFβ). C-DIMS are derivatives of the known natural anticarcinogenic compound 3,3'-bis (indolyl) methane (DIM), and our synthetic derivatives a *p*-substituent phenyl group covalently attached to the methylene bridge connecting the two indole substituents. DIM is a metabolic product in the GI tract (low pH), from the natural compound indole-3-carbinole (I3C) (835, 836). Through luciferase activation assays and BIAcore binding studies, we have demonstrated that some C-DIMs bind NR4A1 and among an initial series of 14 *p*-substituted C-DIMs the *p*-hydroxyphenyl C-DIM analog (DIM-C-pPhOH) bound with high affinity (K_d= 0.11μM) to NR4A1 (803,804). The anticancer activities of DIM-C-pPhOH and related compounds were similar to that observed after knockdown of NR4A1 by RNA interference (RNAi) and these ligands act as NR4A1 antagonists (or inverse agonists) (803-809).

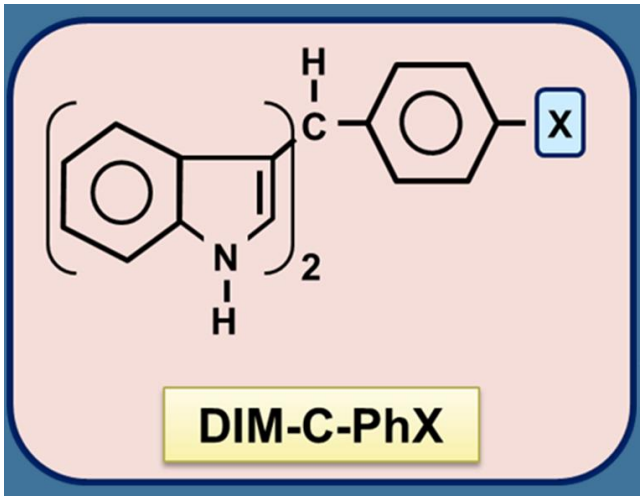

Figure 58: C-DIM structure. Structure of the C-DIM/NR4A1 antagonists, which is composed of two indole substituents connected by a methylene bridge. The methylene bridge also contains a phenyl ring with contains a *para (p)* substituent located at position (X). In this thesis, the following chapters describe the DIM-C-pPhOH (C-DIM 8) C-DIM, where X=OH and the DIM-C-pPhCO$_2$Me (C-DIM 14) C-DIM where X=CO$_2$Me

β1-Integrin and breast cancer

Cell adhesion and attachment are essential for tissue integrity and cellular homeostasis, and the heterodimeric integrin cell surface receptors play a critical role in these processes (369-373,837-838). There are 18 different α and 8 different β subunits that form 24 αβ-integrin receptor heterodimers, and the large 12-member β1-integrin sub-group binds to extracellular matrix (ECM) molecules such as collagen, laminin, fibronectin, tenascin C and vitronectin. Interactions of the integrin receptors with ECM components activate multiple intracellular pathways and induce crosstalk with other signaling systems including EGFR and other receptor tyrosine kinases (369-373, 837-843). The functions of integrin heterodimers are highly tissue-specific and many human pathologies also involve integrin signaling. β1-Integrin is associated with 12 different integrins, with five of these being in breast cancer (α1, α2, α3, α5, α6) (844-860). These bind to collagen, laminin, fibronectin, vitronectin, and tenascin C. β1-Integrin is important for embryonic development, implantation, angiogenesis, and mammary gland development (844-860). In the normal mammary gland, the interaction between the epithelial cells and the basement membrane is critical for the ductal formation and its integrity, which is comprised of a bilayer of luminal epithelial cells and basal myoepithelial cells (849,850). This is segregated by stroma from the basement membrane. Integrin expression is predominant in the myoepithelial layer as this layer directly interacts with the ECM, playing a major role in mammary tissue attachment and integrity, mammary cell growth, development, and differentiation

(849,850). Of these integrins, β1-Integrin has shown to be indispensable for mammary gland longevity as it is a critical component of sustaining mammary gland stem cells (857-869). β1-Integrin also promotes mammary morphogenesis and segregation of mammary ductal compartments (849,85,861,862). Within the mammary gland β1-Integrin, containing heterodimers participate in intracellular-ECM interactions within myoepithelial and alveolar epithelial cells (milk secreting cells). The heterodimers α2β1, α3β1, α6β1 are present in luminal epithelial cell junctions whereas α1β1, α5β1 α6β4 are present predominantly at myoepithelial-basement membrane junctions, with α5β1 demonstrating the highest expression (849,850, 864).

β1-Integrin has been identified as one of the most important integrin receptors in tumorigenesis with prognostic significance and multiple pro-oncogenic functions in several tumor types (853-860). β1-Integrin is highly expressed in most tumors and is associated with a negative prognostic significance such as overall and disease-free survival, recurrence, and metastasis for head and neck and squamous cell carcinoma, melanoma, lung, breast, prostate, laryngeal and pancreatic cancers (857-863). There is now increasing evidence that *β1-integrin* is a pivotal gene involved in tumor growth, survival, adhesion, migration and invasion of cancer cells, and the dominance of one or more of these pathways is dependent on the cell and tumor type and differential expression of other integrin sub-family members. Therapeutics that target integrins have sales in excess of $1.5 billion annually and are being used for

treatment of thrombotic diseases, fibrosis, autoimmune disease, arthritis, age-related macular degeneration and other inflammatory and infectious conditions (864-872). Despite the critical role of β1-integrin in several cancers, therapeutics such as volociximab (monoclonal antibody against α5β1), JSM6427 (small molecule), and ANT-161 (peptide) which inhibits α5β1 binding have made limited clinical impacts for cancer chemotherapy (873-876). β1-integrin has also been correlated with estrogen receptor activation and plays a pivotal role in ER positive breast cancer (877). β1-integrin can control expression of ER and likewise the ER can of α5β1 heterodimer expression (877). This seems to be mediated by kinase LMTK3 (lemur tyrosine kinase 3) (877). Integrin signaling pathways are highly complex in both tumor and non-tumor tissues, and both β1-integrin and focal adhesion kinase (FAK) which is activated by β1-integrin are essential factors in breast cancer cell migration, invasion and metastasis.

A recent immunostaining study of 225 breast invasive ductal carcinomas (IDCs) showed that β1-integrin was overexpressed in 32.8% of patients with IDCs (878). Moreover, there was a correlation between expression of β1-integrin, the oncogene erbB2 and vascular endothelial growth factor (VEGF), and β1-integrin was a prognostic factor for tumor metastasis and decreased survival based on the time between initial diagnosis and death (879,880). Numerous studies demonstrate that FAK is also a negative prognostic factor for breast cancer patients. The important functional role of β1-integrin has been demonstrated in

mouse models, *in vitro* mechanistic studies and by genetic disruption of downstream signaling partners such as FAK and integrin-linked kinase (ILK) and upstream effects induced by fibronectin, tissue factor and L1 cell adhesion molecule (L1CAM) (881,882). For example, in mice expressing erbB2 under the control of the mouse mammary tumor virus and crossed with mammary tissue-specific β1-integrin-deficient mice, there was a decrease in tumor volume, increased apoptosis and decreased lung metastasis compared to animals expressing wild-type β1-integrin (883). There was also a decrease in angiogenic filtration and decreases in activated levels of Src, FAK, p130Cas, and paxillin phosphorylation. There was also a decrease in EGFR phosphorylation, which demonstrates the role that β1-integrin plays in EGFR activation and activation of pro survival PI3K/AKT and Ras pathways (883).

β1-integrin is also involved in Rab mediated endocytic trafficking of EGFR and its reciprocal endocytic recycling of α5β1 heterodimers. Their interactions are important for invasiveness by interacting with the urokinase-type plasminogen activator receptor (884,885). β1-integrin also has an impact on the expression of two other integrins involved in breast cancer invasion: α6 and β4 integrin, which are part of the laminin receptor (886,887). These integrins have been associated with poor prognosis and survival and they promote invasiveness in part by forming a ternary complex with EGFR and promoting Rho activation and invasipodia formation (893-895). This is also critical in ErbB2 overexpressing breast cancer and these pathways all seem to be heavily dependent on β1-integrin (889,890).

β1-integrin has also been studied in mice that have PyVmT-induced carcinogenesis, which represents TNBC (891,892). In these studies, they used a Cre/Lox system to conditionally knockout β1-integrin and demonstrated that β1-integrin was essential for tumor formation in these mice (891,892). Moreover, proliferation of these cells were substantially attenuated as well as Fak localization and activation (891,892). Recent studies also demonstrate that L1CAM and alternatively spliced tissue factor (asTF) enhanced breast cancer cell growth and epithelial to mesenchymal transition (EMT) through activation of β1-integrin (881,882,893). Despite the extensive literature on the pivotal role of β1-integrin in breast cancer growth and metastasis, there is a staggering paucity of drugs that target β1-integrin for breast cancer chemotherapy (873-876). Another problem with drugs that target β1-integrin (including knockdown) is the β1-β3 switching phenomena in which the loss of β1-integrin is accompanied by induction of β3-integrin, another pro-oncogenic integrin (894).

β1-Integrin and pancreatic cancer

β1-Integrin mRNA and protein are overexpressed in pancreatic tumors, and β1-integrin silencing by RNA interference (RNAi) in pancreatic cancer cells decreases cell adhesion, migration and invasion, and downregulation of genes such as *MMP-2* and *MMP-9* (895-899). Knockdown of β1-integrin in Colo-357 pancreatic cancer cells by RNAi also decreases expression of α2-, α3-, α5- and αv-integrins; these same integrins form heterodimers with β1-integrin and bind

collagen (α2), laminin (α3), and fibronectin (α5 and αv) (895-899). β1-Intregrin silencing by RNA interference (RNAi) also decreases tumor growth and metastasis, and similar results have been observed *in vivo* using β1-integrin antibodies (900,901). Rip1Tag2 transgenic mice express the Simian Virus 40 large T-antigen under the control of the rat insulin promoter and spontaneously develop pancreatic β-cell tumors, and ablation of β1-integrin in tumor cells decreases tumor cell growth and metastasis (902,903).

Pancreatic cancer cells express β1- and other α-integrin partners and, not surprisingly, β1-integrin heterodimers are activated by multiple extracellular matrix proteins (e.g. collagens, fibronectin, laminin, vitronectin) and many other extracellular factors, including tissue factor (TF) and alternatively spliced TF (asTF) which are overexpressed in pancreatic tumors (904-906) and exhibit potent growth promoting, angiogenic, and metastatic activity (907-910). The L1 cell adhesion molecule (L1CAM) is a transmembrane glycoprotein (200-220 kDA) that is overexpressed in pancreatic ductal adenocarcinoma cells (911). L1CAM-mediated drug-resistance, survival, and epithelial to mesenchymal transition (EMT) in pancreatic cancer cells is due to interactions of α5 and β1-integrins and the immediate downstream targets include FAK, integrin-linked kinase (ILK) and PI3-K (912-916). L1CAM-β1-integrin activation is associated with induction of IL-1β and activation of NFκB (914-916), and this signaling pathway "supports a motile and invasive tumor cell phenotype" (915). Other factors including

neuropilin-1, glial cell line-derived neurotrophic factor, thrombin, Snail, Slug and interleukin β either interact with or enhance β1-integrin and thereby modulate downstream signaling in pancreatic cancer cells (917-921).

β1-Integrin and colon cancer

β1-integrin is also overexpressed in colon cancer and is critical for attachment of colon cancer cells to blood vessels, intravasation, and metastasis (922-924). Knockdown of β1-integrin using RNAi in colon cancer cells results in decreased cancer cell growth migration and induces apoptosis. High expression of α5β1 in colon cancer cells promotes malignant progression in colon cancer (922-924). A recent report showed that E-selectin mediated increase in cancer cell attachment to vascular endothelium is due to upregulation of β1-integrin (922). Upregulation of β1-integrin in colon cancers also increase endotoxin levels that enhance association to laminin in endothelial cells (923). Increased expression of β1-integrin or α5 integrin in HT29 colon cancer cells suppresses nutrient starvation triggered apoptosis (924). Studies by our group has demonstrated that NR4A1 is overexpressed in colorectal cancer and that it is an important driver in oncogenesis, invasion, reduction of oxidative stress, and inhibitor of apoptosis. Recently we have demonstrated that NR4A1 mediates cancer cell migration which is also β1-integrin-dependent and our studies (chapter VII) demonstrate for the first time that NR4A1 regulates β1-integrin gene expression and C-DIMs/NR4A1 antagonists inhibit β1-integrin expression without inducing β3-integrin.

CHAPTER II

NUCLEAR RECEPTOR 4A1 (NR4A1) AS A DRUG TARGET FOR RENAL CELL ADENOCARCINOMA

Introduction

Kidney cancer is a complex and heterogenous disease and clear cell renal cell adenocarcinoma (RCC) is the most common sub-type and represents approximately 75% of renal parenchymal tumors (924-926). The incidence of RCC has been increasing and it is estimated that in 2013 over 65,000 new cases will be diagnosed and 13,680 kidney cancer patients will die from this disease (927). Early stage RCC patients with localized tumors have a good prognosis after surgical removable of the primary tumor; however, approximately 30% of all patients first diagnosed with RCC already have metastatic disease (924). Patients with RCC are unusually resistant to radio and cytotoxic drug therapies compared to responses observed for other solid tumors, and immunotherapies have provided some limited benefits for patients with RCC metastases (928-931). The recent development of targeted therapies for treating RCC has significantly improved the outlook for patients and the focus has primarily been on clinical application of receptor tyrosine kinase inhibitors, neutralizing antibodies against vascular endothelial growth factor (VEGF) and mTOR pathway inhibitors (932-936).

Studies in this laboratory have identified 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl)methanes (C-DIMs) as novel antineoplastic agents that act as antagonists of the orphan nuclear receptor 4A1 (NR4A1, TR3, Nur77) which exhibits pro-oncogenic activity in several solid tumors (803,805,806,937). For example, the *p*-hydroxyphenyl C-DIM analog (DIM-C-pPhOH) binds nuclear NR4A1, and the effects on downstream genes and pathways are similar to that observed after knockdown of NR4A1 by RNA interference (RNAi). Results of these studies in pancreatic, lung and colon cancer cell lines show that NR4A1 regulates at least three pathways that are important for cancer cell proliferation and survival (Fig. 1A). NR4A1 regulates expression of pro-survival (survivin, bcl-2) and growth promoting (EGFR) genes through interaction with Sp1 bound to their corresponding proximal GC-rich promoters (804). NR4A1 binds to and inactivates p53 and p53-regulated sestrin 2 which results in activation of mTOR in cells expressing NR4A1 and wild-type p53. NR4A1 also regulates expression of genes such as thioredoxin domain containing 5 (TXNDC5) and isocytrate dehydrogenase 1 (IDH1) to maintain low oxidative stress in cancer cells (805). Thus, inactivation of NR4A1 by the receptor antagonist DIM-C-pPhOH decreases expression of genes associated with cell proliferation and survival, induces oxidative stress, and inhibits mTOR in cancer cell lines (806).

In this study, we show that NR4A1 is also expressed in the ACHN and 786-O kidney cancer cell lines and treatment of these cells with DIM-C-pPhOH and the related NR4A1 antagonist 1,1-bis(3'-indolyl)-1-(*p*-

carboxymethylphenyl)methane (DIM-C-pPhCO_2Me) inhibits cell growth, induces apoptosis and cellular stress, and inhibits mTOR signaling. A comparison of the effects of the NR4A1 antagonists and knockdown of NR4A1 by RNAi (siNR4A1) gave comparable results and inactivation of the previously characterized pro-oncogenic NR4A1-regulated pathways (Fig. 1A). These results suggest that NR4A1 is a potential drug target for treating RCC patients that overexpress this receptor.

Materials and Methods

Cell lines and antibodies

ACHN and 786-0 human kidney cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained 37˚C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic or RPMI-1640 Medium with 10% fetal bovine serum and antibiotic respectively. B-actin antibody Dulbecco's Modified Eagle's Medium, and RPMI-1640 Medium were purchased from Sigma-Aldrich (St. Louis, MO). Sp1 antibody and Glutathione (GSH) reduced free acid were purchased from Millipore (Temecula, CA); sestrin 2 (SESN2), bcl2, CHOP, ATF4, isocitrate dehydrogenase 1 (IDH1), and epidermal growth factor receptor (EGFR) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Nur77(D63C5) XP®, caspase 3, cleaved poly ADP ribose polymerase (c-PARP;

9541), phospho mTOR, mTOR, phospho AMPKα, AMPKα, phospho p70S6K, p70S6K, phospho S6RP, S6RP, phospho 4EBP1, 4EBP1, and survivin antibodies were purchased from Cell Signaling technologies (Danvers, MA). TXNDC5 antibody was purchased from Genetex (Irvine, CA). Phospho PERK antibody was obtained from Biolegend (San Diego, CA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA). Cells were visualized under an EVOS fl, Fluorescence microscope, from Advanced Microscopy Group using a multiband filter set for FITC, rhodamine, and DAPI. The C-DIM compounds were prepared as previously described (942-944).

Cell proliferation assay

ACHN and 786-0 kidney cancer cells (1.0×10^5 per well) were plated in 12 well plates and allowed to attach for 24 hours and cells were treated with DIM-C-pPhOH or DIM-C-pPhCO_2Me (dimethyl sulfoxide, DMSO, as empty vehicle) for 24 or 48 hours or by siNR4A1(iGL2 as control siRNA with lipofectamine vehicle) for 72 hours. Cells were then trypsinized and counted after respective treatment time intervals using a Coulter Z1 cell counter and growth inhibition was determined. Each experiment was carried out in triplicate, and results were expressed as the mean ± SE for each set of experiments.

Annexin V staining

ACHN and 786-0 kidney cancer cells(1.0×10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from

Thermo Scientific and were allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or DIM-C-pPhCO$_2$Me (15 µM) was added for 24 hr. For siRNA treatment, cells were treated with iGL2 or 100 nm siNR4A1 (1 or 2) siRNA for 72 hours. Apoptosis was analyzed by apoptotic and necrotic assay kit (Biotium CA), which contained fluorescein isothiocyanate-annexin-V, ethidium homodimer III and Hoechst 3342. Apoptosis, necrotic and healthy cell detection kit was used according to the manufacturer's protocol and were visualized under an EVOS fl, fluorescence microscope, from Advanced Microscopy. The proportion of apoptotic cells was determined by the amount of green fluorescence observed in the treatment groups relative and normalized to control group.

Immunofluorescence

ACHN and 786-0 cells (1.0 x 10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or DIM-C-pPhCO$_2$Me (15 µM) was added for 24 hr. Cells were then treated with fluorescent NR4A1 primary antibody (Nur77 (D63C5)) XP®) and immunofluorescence was observed according to Cell Signaling Technology's immunofluorescence protocol. DAPI staining was observed using Hoechst staining according to Biotium's apoptotic and Necrotic assay kit following the manufacturers protocol. Cells were visualized by microscope (Advanced

Microscopy). NR4A1 localization was determined by green fluorescence. DAPI was used to stain the nucleus. Images were taken sequentially of NR4A1, DAPI, and then merged.

Western blot analysis

ACHN and 786-0 kidney cancer cell lines (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. Cells were seeded and subsequently treated with varying concentration of DIM-C-pPhOH or DIM-C-pPhCO_2Me for 24 hours or with 100 nm of siNR4A1 for 72 hours. Cells were lysed with high salt lysis buffer (with protease inhibitor cocktail) and quantitated with Bradford reagent. Lysates were then analyzed by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane by wet electroblotting. Membranes were then incubated with primary and then followed by secondary antibody. Western blot analysis was determined as described and Immobilon western chemiluminescence substrates (Millipore, Billerica, MA) were used to develop images captured on a Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR).

Small interfering RNA interference assay

ACHN and 786-0 kidney cancer cells were seeded (1.2×10^5 per well) in six well plates in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and left to attach

for 24 hours. Knockdown of NR4A1 was carried out using LIpofectamine 2000 reagent according to the manufactures protocol. Small inhibitory RNAs and GL2 (non-specific oligonucleotide) were prepared and purchased Sigma-Aldrich (St. Louis MO). The siRNA complexes used in the study are as follows: siGL2-5', CGU ACG CGG AAU ACU UCG A; siNR4A1 (1)-SASI_Hs02_00333289; siNR4A1 (2)-SASI_Hs01_00182072

Generation and measurement of ROS

Cellular ROS levels were ascertained using the cell permeable probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2'7' dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen (Grand Island, NY). CM-H_2DCFDA is non-fluorescent until cleavage of the acetyl groups by intracellular esterases and oxidation that transpires within the cell. Following treatment of the cells for 12 or 24 hours with DIM-C-pPhOH, DIM-C-pPHCO_2Me or siNR4A1 for 72 hours, cells plated on a 6-well culture plate were trypsinized, neutralized, then loaded with 10µM of probe for 20 min, washed once with serum free medium, and then ROS was measured by flow cytometry using Accuri's C6 Flow Cytometer (Accuri, Ann Arbor, MI).

Orthotopic Xenograft model

Male athymic nude mice (Foxn1nu, aged 6–7 weeks) were purchased from Harlan (Indianapolis, IN). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions. A xenograft was established by

subcutaneous injection of cultured ACHN cells (10^7 cells/150 µl) with Matrigel into the flanks of individual mice. Tumors were allowed to grow for 7 days until tumors were palpable. Mice were then randomized into two groups of five mice per group and dosed by oral gavage with either corn oil or DIM-C-pPHOH (30 mg/kg/day) for 50 days. The mice were weighed, and tumor size was measured twice a week with calipers to permit calculation of tumor volumes, $VxLxW^2/2$, where L and W were length and width. Tumor lysates were obtained and analyzed for protein expression by western blots.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by student's *t* test. The results are expressed as means with error bars representing 95% confidence intervals for 3 experiments for each group unless otherwise indicated, and a *P* value less than 0.05 was considered statistically significant. All Statistical tests were 2-sided.

Results

NR4A1 antagonists inhibit RCC cell proliferation and induce apoptosis

Figure 59.

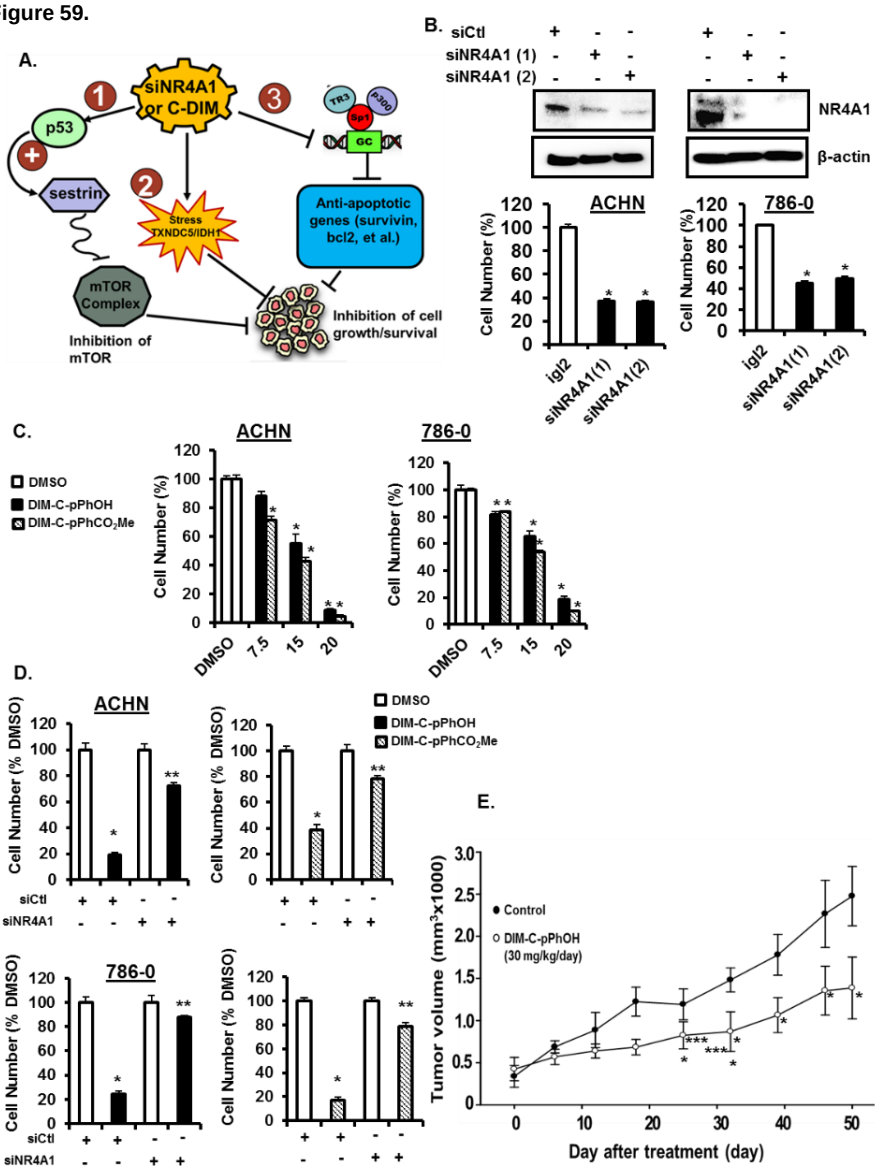

Figure 59. NR4A1 plays a role in RCC proliferation. (A) Pathways activated by NR4A1 and targeted by NR4A1 antagonists. (B) Cells were transfected with two different oligonucleotides targeting NR4A1 [NR4A1(1) and NR4A1(2)] and after 72 hr, the number of cells were determined as outlined in the Materials and Methods. (C) Cells were treated with DIM-C-pPhOH and DIM-C-pPhCO$_2$Me and cell numbers were determined after treatment for 24 hr. (D) Cells were transfected with siCtl (non-specific) or siNR4A1 and treated with DIM-C-pPhOH and DIM-C-pPhCO$_2$Me and cell numbers were determined. siNR4A1 alone decreased cell proliferation as indicated in (B) and this value was set at 100% to determine the effects of C-DIMs in cells after loss of NR4A1. (E) Athymic nude mice bearing ACHN cells as xenografts were treated with 30 mg/kg/d DIM-C-pPhOH and tumor volumes were determined. Results are means ± SE for at least 3 separate determinations and significantly ($p < 0.05$) decreased growth/volume is indicated (*). Significant attenuation of C-DIM-induced growth after NR4A1 is also indicated (**).

Figure 59A summarizes the growth and survival pthways that can be targetted by NR4A1 antagonists in lung pancreatic and colon cancers. ACHN and 786-O RCC cell lines are p53-positive and mutant cell lines, respectively, and were used to investigate the function of NR4A1 and effects of C-DIM/NR4A1 antagonists. In cells transfected with two different oligonucleotides that target NR4A1 (siNR4A1), there was a significant 50-60% decrease in proliferation of both cell lines (Fig. 59B). Moreover, treatment of ACHN and 786-O cells for 24 hr with 0-20 μM DIM-C-pPhOH or DIM-C-pPhCO_2Me resulted in a significant decrease in cell proliferation (Fig. 59C), and IC_{50} values for both compounds in ACHN cells were 13.6 and 11.7 μM, respectively, and in 786-O cells the values were 13.0 and 13.4 μM, respectively. The role of NR4A1 in mediating the growth inhibitory effects of DIM-C-pPhOH and DIM-C-pPhCO_2Me was further investigated by treating ACHN cells with C-DIMs after knockdown of NR4A1 by RNAi. The results show that the magnitude of the growth inhibitory effects of both compounds was significantly attenuated in cells in which NR4A1 was decreased, thus confirming a role for NR4A1 in mediating this response (Fig. 59D). Moreover, treatment of athymic nude mice bearing ACHN cells as xenografts with DIM-C-pPhOH (30 mg/kg/d) for 50 days also resulted in a significant inhibition of tumor growth (Fig. 59E) and this complemented results of the *in vitro* studies. Thus, both knockdown of NR4A1 by RNAi or treatment with C-DIM/NR4A1 antagonists inhibited RCC cell and tumor growth. Transfection of ACHN and 786-O cells with two different siNR4A1 oligonucleotides also increased Annexin V staining (Figs.

60A and 60B) ,which is a marker of apoptosis, and induced cleavage of caspases7, 8 and PARP (Suppl. Figs. A-1A and A1-B). We also observed that both DIM-C-pPhOH and DIM-C-pPHCO$_2$Me induced Annexin V staining in ACHN and 786-O cells (Figs. 60C and 60D, respectively), and cleavage of caspase7, 8 and PARP (Suppl. Fig. A-1C and A-1D) confirming that C-DIM/NR4A1 antagonists induce apoptosis in RCC cell lines.

Figure 60.

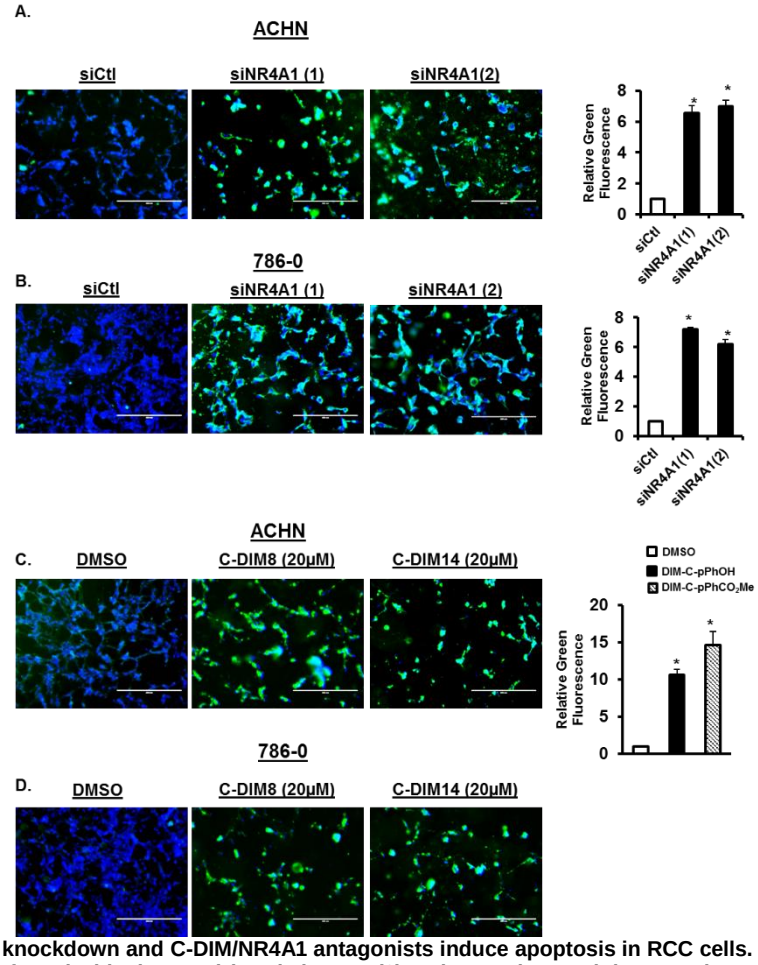

Figure 60. NR4A1 knockdown and C-DIM/NR4A1 antagonists induce apoptosis in RCC cells. ACHN (A) or 786-O (B) cells were transfected with siNR4A1(1) and siNR4A1(2) and Annexin V staining was determined as outlined in the Materials and Methods. ACHN (C) and 786-O (D) cells were treated with 20 μM DIM-C-pPhOH or DIM-C-pPhCO$_2$Me for 24 hr and Annexin V staining was determined. Results are means ± SE for 3 replicated determinations and significant ($p<0.05$) induction of Annexin V staining is indicated (*).

Previous studies show that many apoptosis inducers that act through NR4A1 induce nuclear export of the receptor, which subsequently forms a pro-apoptotic complex with the mitochondrial bcl-2 protein (906,916,938). In contrast, our studies show that C-DIMs act through nuclear NR4A1 in cancer cells (803-806). ACHN and 786-O cells were treated with DIM-C-pPhOH and DIM-C-pPHCO$_2$Me and after 24 hr, cells were stained with NR4A1 antibodies and DAP1 and the results show that DAP1 and the NR4A1 immunostaining were co-localized in the nucleus, demonstrating that the C-DIM/NR4A1 antagonists act through the nuclear receptor (Fig. 61).

Figure 61.

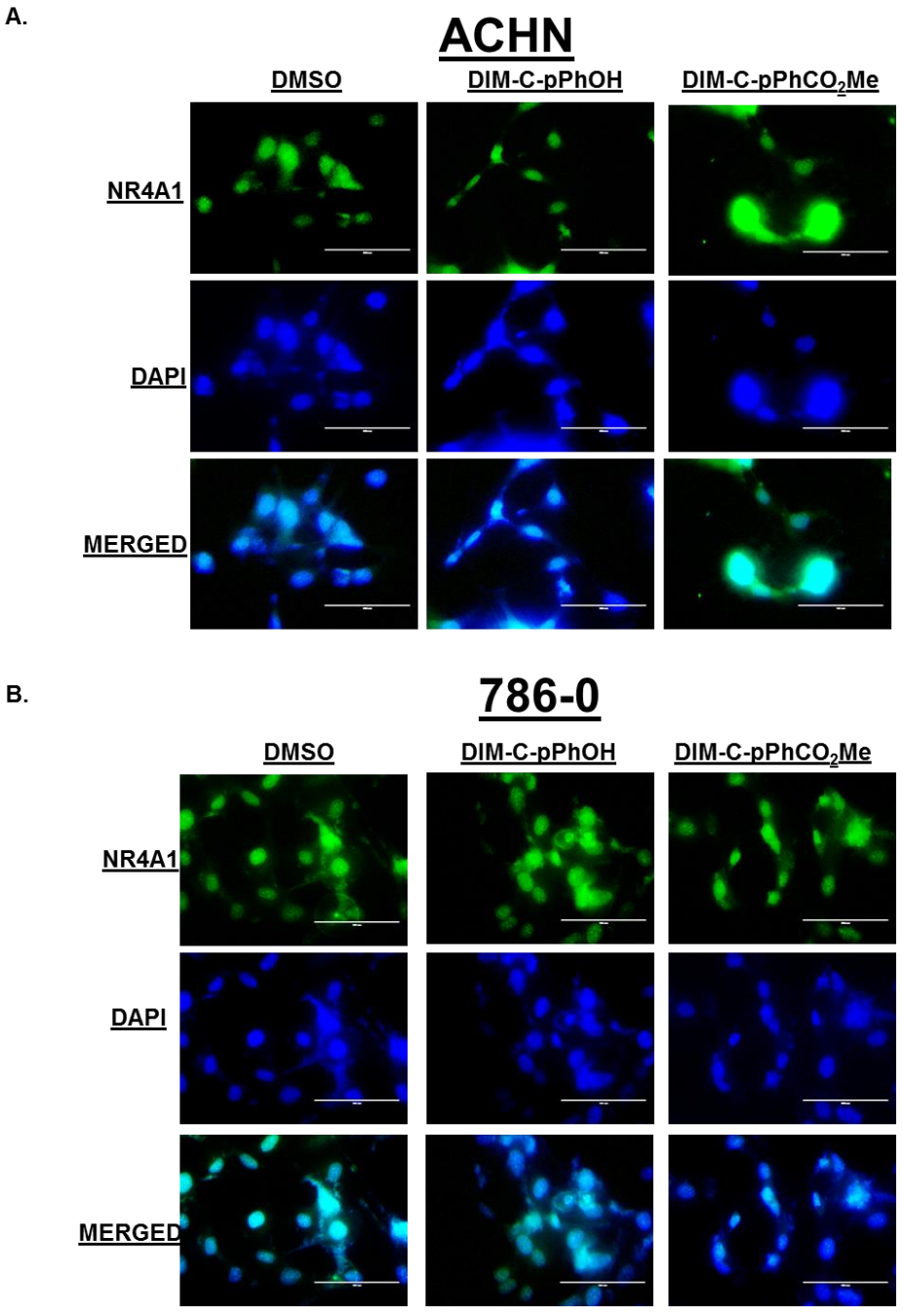

Figure 61. C-DIM/NR4A1 antagonists target nuclear NR4A1. ACHN (A) and 786-O (B) cells were treated with 20 μM DIM-C-pPhOH and DIM-C-pPhCO$_2$Me. Cells were immunostained with NR4A1 antibodies or DAPI and images merged as outlined in the Materials and Methods.

Sp-regulated survival genes

Previous studies showed that NR4A1 in combination with p300 activated Sp-regulated genes such as survivin, bcl-2 and EGFR in cancer cells (803-806). Transfection of ACHN and 786-O cells with siNR4A1 decreased expression of survivin, bcl-2 and EGFR and this was accompanied by increased PARP cleavage (primarily in ACHN cells), a marker of apoptosis (Fig. 62A). Similar results were observed in both RCC cell lines after treatment with DIM-C-pPhOH (Fig. 62B) or DIM-C-pPhCO$_2$Me (Fig. 62C), confirming that the NR4A1 antagonists inhibited NR4A1-regulated expression of survivin, bcl-2 and EGFR in ACHN and 786-O cells as previously reported in pancreatic, lung and colon cancers (803-805). In addition, we also observed decreased expression of survivin, bcl-2, EGFR and induced PARP cleavage in tumor lysates from nude mice bearing ACHN cells as a xenograft and treated with DIM-C-pPhOH (30 mg/kg/d) (Fig. 62D).

Figure 62.

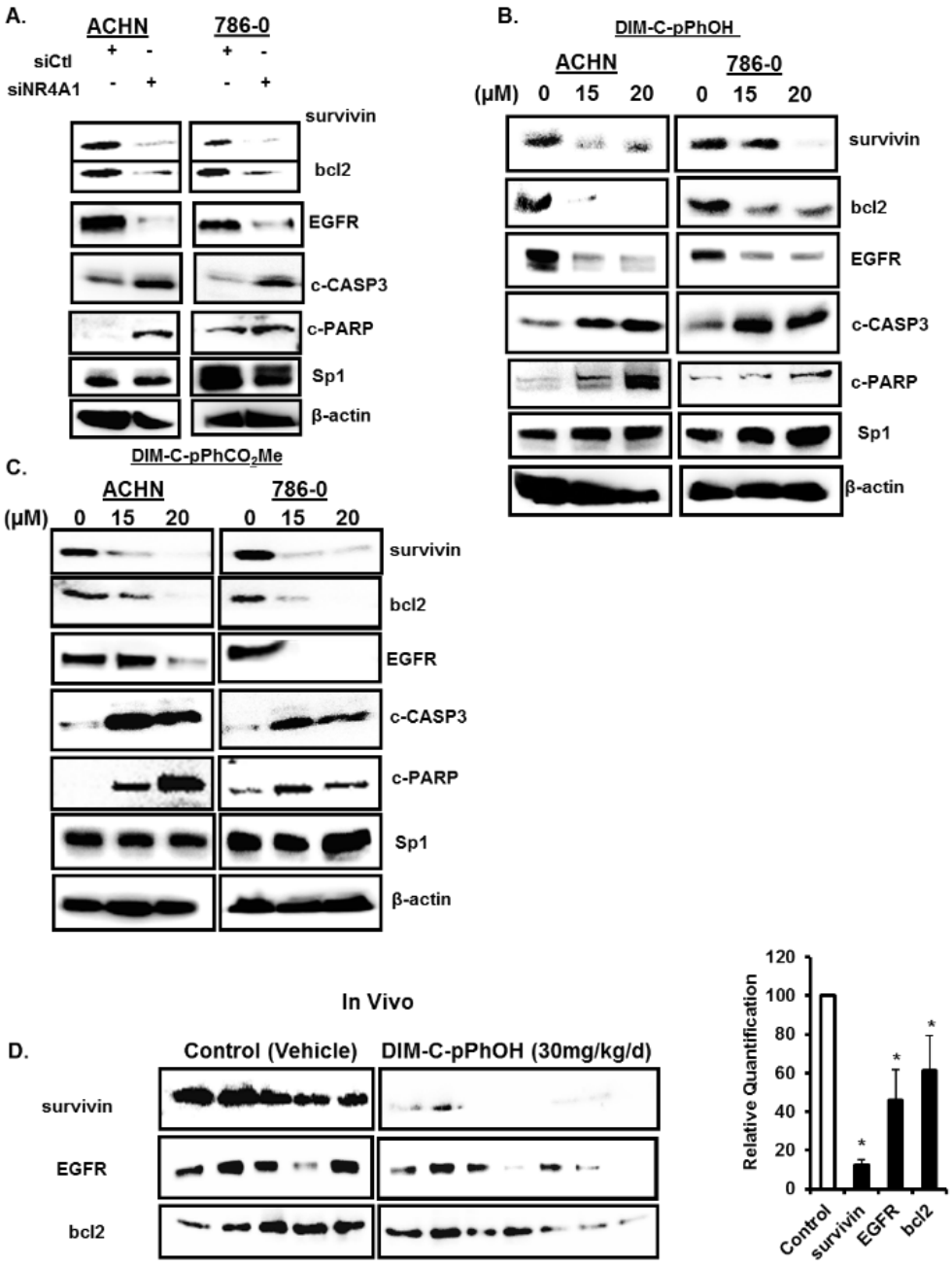

Figure 62. NR4A1 coregulates Sp-regulated oncogenic genes. (A) Cells were transfected with siNR4A1 and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. Cells were treated with DIM-C-pPhOH (B) or DIM-C-pPhCO$_2$Me (C) and after 24 hr, whole cell lysates were analyzed by western blots. (D) Western blot analysis of tumor lysates from athymic nude mice bearing ACHN xenografts and treated with vehicle (control) or DIM-C-pPhOH (30 mg/kg/d) was also determined. Band intensities were quantitated relative to β-actin (means ± SE) and significantly decreased staining intensities are indicated (*; p<0.05).

siNR4A1 and C-DIM/NR4A1 antagonist induce stress in RCC cells

It has been shown that NR4A1 maintains low levels of stress in cancer cells by regulating expression of TXNDC5 and IDH1 (Fig. 59A) that in turn maintain high levels of cellular reducing agents (805,806). Transfection of ACHN and 786-O cells with siNR4A1 decreased expression of TXNDC5 and IDH1 in both cell lines and this was accompanied by increased expression of CHOP, ATF4 and phospho-PERK which are markers of ER stress (Fig. 63A). Treatment of ACHN and 786-O cells with DIM-C-pPhOH (Fig. 63B) or DIM-C-pPhCO_2Me (Fig. 63C) also decreased expression of TXNDC5 and IDH1 and induced markers of ER stress (CHOP, ATF4 and p-PERK). Moreover, siNR4A or treatment with the C-DIM/NR4A1 antagonists also induced ROS as determined using the cell permeant probe CM-H_2-DFDA (Fig. 63D). In addition, DIM-C-pPhOH (30 mg/kg/d) also decreased expression of TXNDC5 and IDH-1 and induced CHOP expression in tumors from athymic nude mouse xenografts (Fig. 63E), demonstrating that C-DIM/NR4A1 antagonists-dependent inhibition of RCC cell and tumor growth is due, in part, to induction of stress (Fig. 59A).

Figure 63.

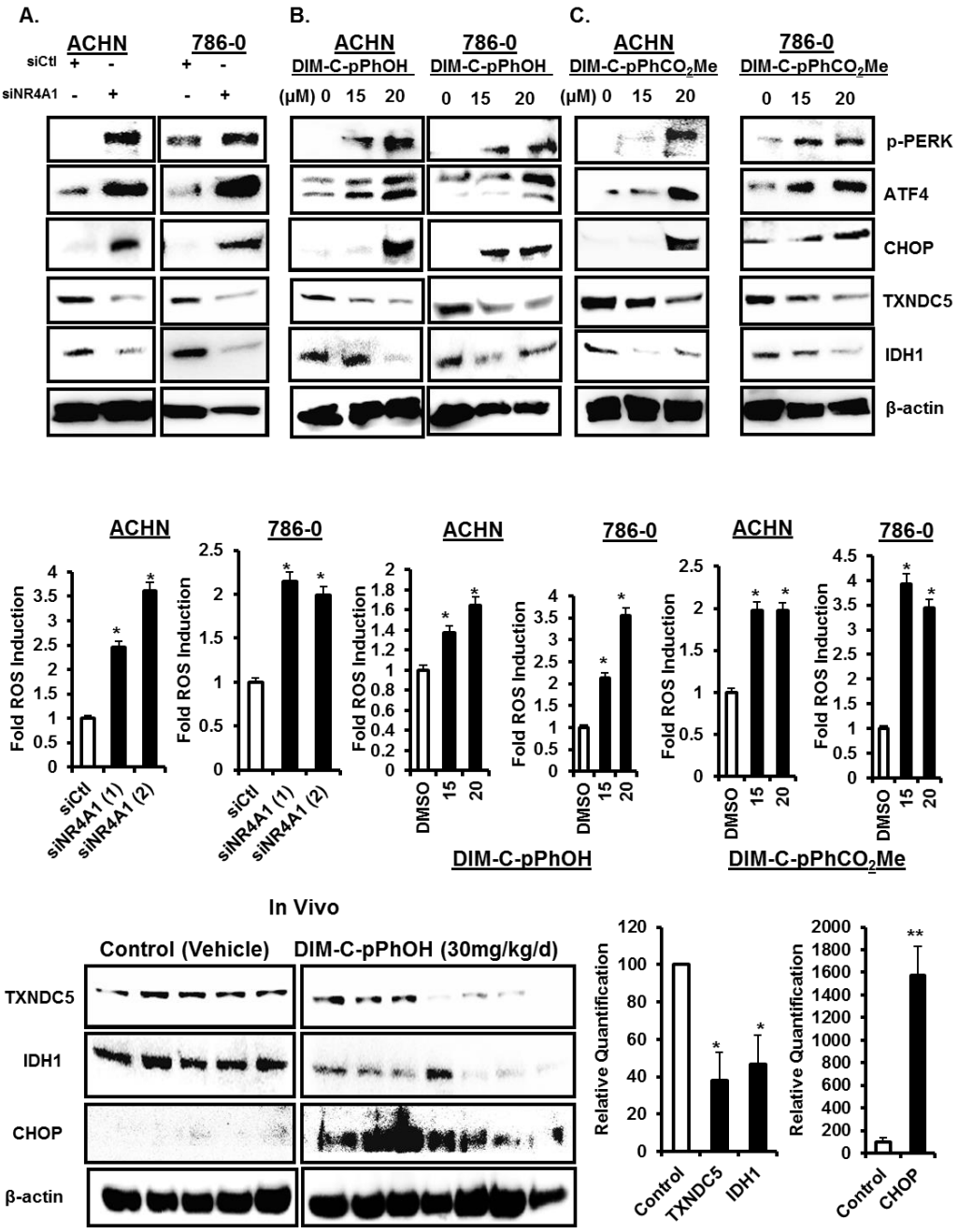

Figure 63. siNR4A1 and C-DIM/NR4A1 antagonists induce stress in RCC cells. Cells were transfected with siNR4A1 (A) or treated with DIM-C-pPhOH (B) and DIM-C-pPhCO$_2$Me (C) and whole cell lysates were analyzed by western blots. (D) After treatment of cells as described in (A) - (C), ROS was measured using the cell permeant CM-H2FDA probe as outlined in the Materials and Methods. Significant induction ($p<0.05$) of ROS is indicated (*). Tumor lysates from athymic nude mice bearing ACHN cells as xenografts and treated with vehicle control or DIM-C-pPhCO$_2$Me were analyzed by western blots and individual bands were quantitated (relative to β-actin). Significant ($p<0.05$) induction of ROS is indicated (*).

siNR4A1 and C-DIM/NR4A1 antagonists inhibit mTOR

NR4A1 binds and inactivates p53 (939) and treatment with siNR4A1 or C-DIM/NR4A1 antagonists results in p53-dependent induction of sestrin 2 which activates AMPKα and inhibits mTOR (803,806). Knockdown of NR4A1 by RNAi in ACHN cells that express wild-type p53 resulted in the induction of sestrin 2, activation of AMPKα, and inhibition of phospho-mTOR; this was also accompanied by decreased activation/phosphorylation of mTOR-regulated p70S6K, 6SRP and 4EBP1 (Fig. 64A). Treatment of ACHN cells with the NR4A1 antagonists DIM-C-pPhOH (Fig. 6B) and DIM-C-pPhCO_2Me (Fig. 64C) also induced sestrin 2, activated AMPKα and inhibited activation of mTOR and downstream kinases and these results are consistent with previous studies in other cancer cells expressing wild-type p53 (803,806). The 786-O cell line expresses a mutant p53; however, transfection of these cells with siNR4A1 also resulted in the induction of sestrin 2 and inactivation of mTOR and mTOR signaling (Fig. 65A). Moreover, similar results were observed after treatment of 786-O cells with the NR4A1 antagonists DIM-C-pPhOH (Fig. 65B) and DIM-C-pPhCO_2Me (Fig. 65C). It has previously been reported that sestrin 2 is induced by other factors including oxidative stress (940), and Figure 7D shows that induction of sestrin 2 by siNR4A1, DIM-C-pPhOH and DIM-C-pPhCO_2Me was attenuated after cotreatment with the antioxidant glutathione (GSH). Thus, induction of ROS by NR4A1 antagonist-mediated downregulation of TXNDC5 and IDH1 also results in the induction of sestrin 2 in cells expressing mutant p53,

suggesting that NR4A1 antagonists inhibit mTOR by both p53-dependent and -independent induction of sestrin2. These studies demonstrate that NR4A1 is a target for NR4A1 antagonists in RCC cells; this leads to inhibition of several NR4A1-regulated pro-oncogenic pathways (Fig. 1A) including mTOR and these results are comparable to those observed in pancreatic, lung and colon cancer cells and tumors (803-806).

Figure 64.

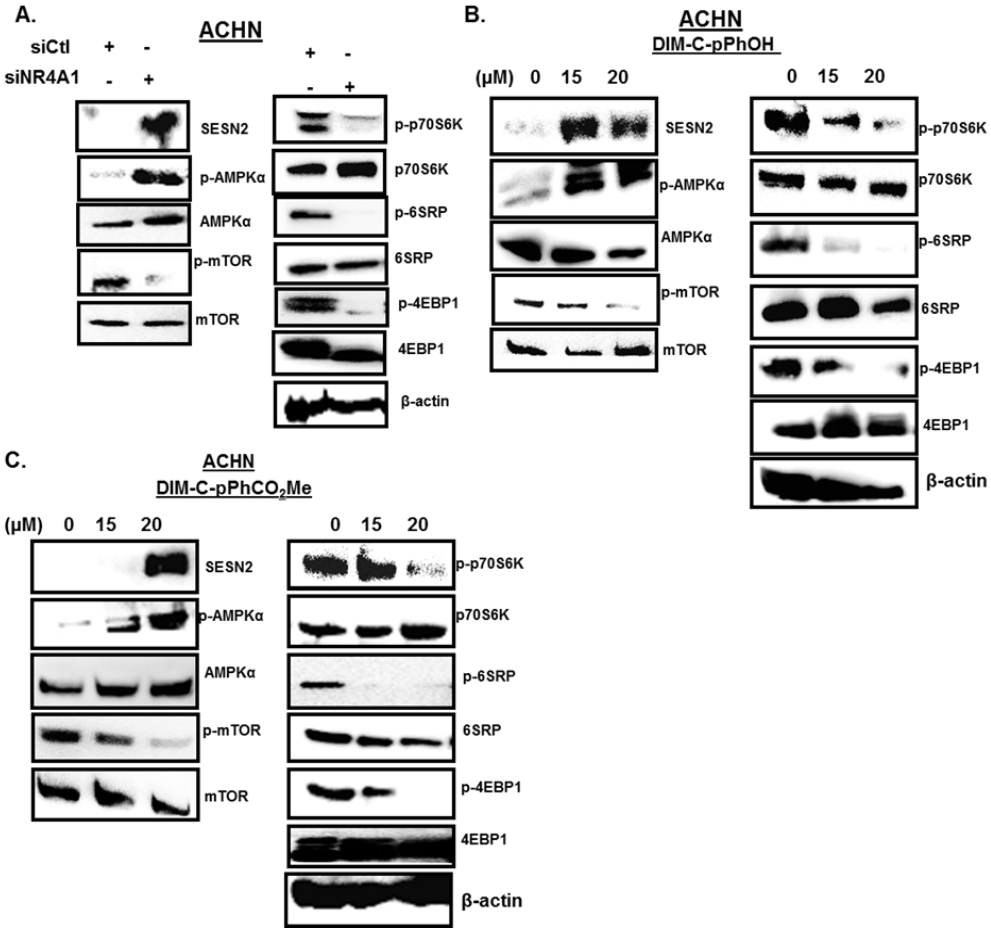

Figure 64. siNR4A1 and NR4A1 antagonist inhibit mTOR in ACHN cells. Cells were transfected with siNR4A1 (A) and treated with DIMI-C-pPhOH (B) and DIM-C-pPhCO$_2$Me (C), and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods.

Figure 65.

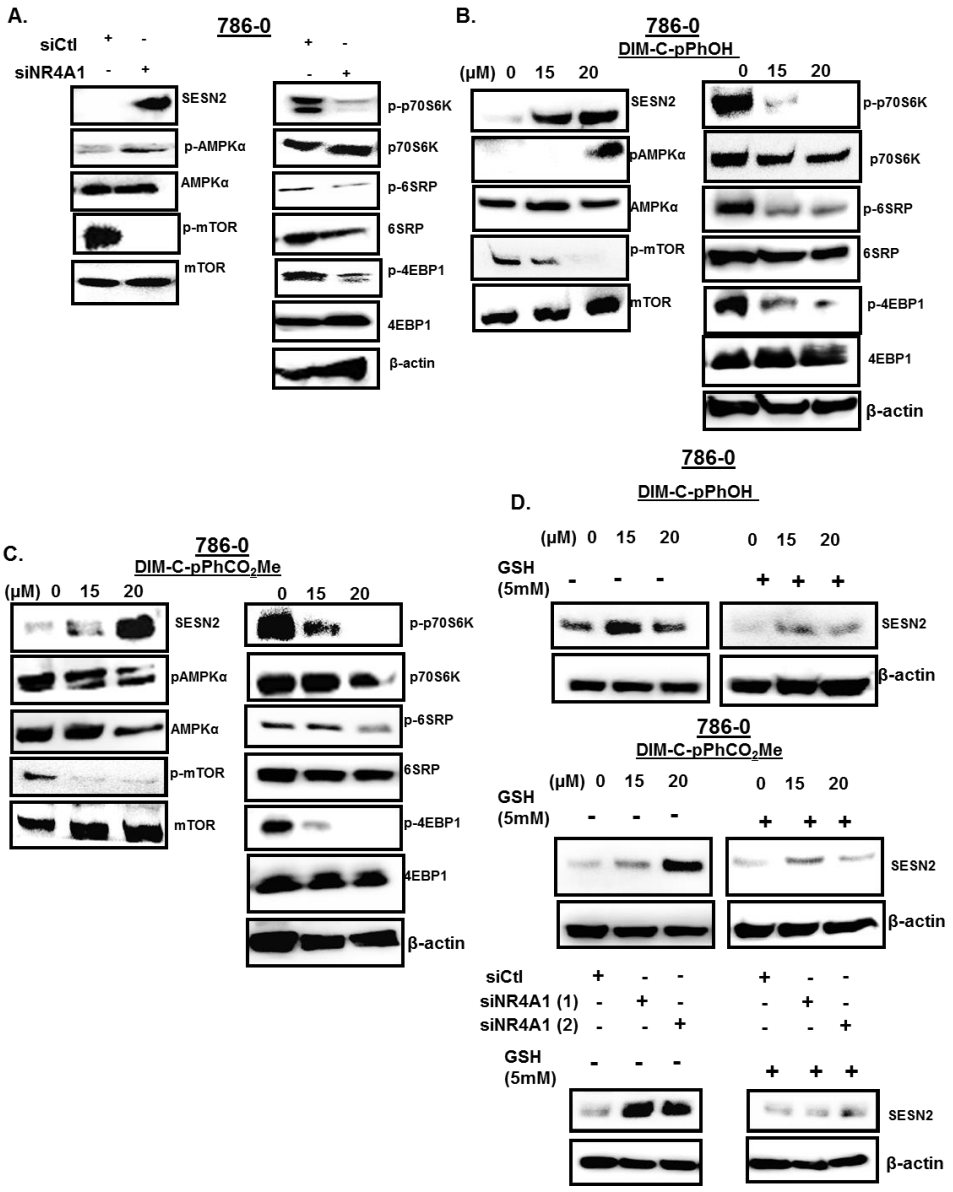

Figure 65. siNR4A1 and NR4A1 antagonist inhibit mTOR in 786-0 cells. Cells were transfected with siNR4A1 (A) and treated with DIMI-C-pPhOH (B) and DIM-C-pPhCO$_2$Me (C), and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (D) Induction of sestrin 2 by western blots was determined in 780-O cells treated with DIM-C-pPhOH and DIM-C-pPhCO$_2$Me or transfected with siNR4A1 in the presence or absence of 5 mM GSH.

Discussion

NR4A receptors are immediate-early genes that are induced by diverse stressors in multiple tissues/cell lines and these receptors play increasingly important role in maintaining cellular homeostasis (891,892). There is evidence that NR4A receptors play a role in the central nervous system, inflammation, cardiovascular function, T cell and macrophage function, and metabolic function; these receptors also play a role in carcinogenesis and other diseases. The function of NR4A1 in cancer cells has been extensively investigated and in solid tumors, there is evidence that this receptor exhibits unique functions that are dependent on its subcellular location (788, 808,905). NR4A1 is a nuclear receptor; however, treatment of some cancer cell lines with apoptosis-inducing agents results in the induction and nuclear export of NR4A1, which associates with mitochondrial bcl-2 to form a pro-apoptotic complex (905,906,916, 938). Thus, mitochondrial NR4A1 exhibits tumor suppressor-like activity, and both paclitaxel and peptide mimetics of NR4A1 that bind bcl-2, also activate apoptosis in cancer cells (906,907). In contrast, results of RNAi studies suggest that nuclear NR4A1 is pro-oncogenic and plays a role in cancer cell proliferation and survival, and studies in this laboratory have identified at least three pathways and associated genes that contribute to the functions of NR4A1 in cancer cells (Fig. 59A). Thus, NR4A1 is unique among orphan nuclear receptors, and the observed pro-oncogenic or tumor suppressor-like activity is dependent on the subcellular location of the receptor. Several different structural classes of ligands bind NR4A1

and these include cytosporone B and related analogs (910,911), C-DIMs (806), ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl]acetate (TMPA) (941), and 1-(3,4,5-trihydroxyphenyl)nonan-1-one (942). Cytosporone B and related compounds and 1-(3,4,5-trihydroxyphenyl)nonan-1-one induce nuclear export of NR4A1 and TMPA antagonizes nuclear NR4A1-LKB1 interactions but does not affect NR4A1-dependent transactivation. In contrast, our previous studies in pancreatic, lung and colon cancer cells show that C-DIM/NR4A1 antagonists act on nuclear NR4A1 and do not induce nuclear export of the receptor [confirmed in RCC cells (Fig. 3)] and these compounds also decrease NR4A1-dependent transactivation (803,805,806,937). These C-DIMs also require NR4A1 for inhibitory action as observed for cell proliferation in Supplemenatry Figure A-2

Kidney injury can induce NR4A1 expression in non-tumor tissue, and one study reported the NR4A1 was expressed in 786-O cells and NR4A1 mRNA was more highly expressed in tumors from patients with RCC compared to surrounding non-tumor tissue (943-945). Currently, we are accumulating tumors from kidney cancer patients to investigate the tumor-type specific expression and prognostic significance of NR4A1. We further investigated the pro-oncogenic functions of NR4A1 by RNAi and compared the results with that observed after treatment of ACHN or 786-O cells with two prototypical NR4A1 antagonists, DIM-C-pPhOH and DIM-C-pPhCO_2Me. Both NR4A1 knockdown and the NR4A1 antagonists inhibited ACHN and 786-O cell proliferation, and DIM-C-pPhOH also inhibited tumor growth in athymic nude mice bearing ACHN cells as xenografts (Fig. 59)

and siNR4A1 and NR4A1 antagonists also induced apoptosis in these cell lines (Fig. 60). These effects are comparable to previous studies in pancreatic, lung and colon cancer cell lines (803-806), suggesting the potential therapeutic potential for C-DIMs in treating RCC in patients that overexpress NR4A1.

Figure 59A illustrates three pro-oncogenic pathways and related genes that are regulated by NR4A1 in pancreatic, lung and colon cancer cell lines, and we have also observed these NR4A1-dependent response/pathways in other cancer cell lines (data not shown). NR4A1 in combination with p300 coactivates survivin and other Sp1-regulated genes (804), and results in Figure 4 show that survivin, bcl-2 and the EGFR were regulated by NR4A1 in RCC cells. All three of these Sp-regulated genes play a role in cancer cell growth and survival and some studies show that expression of survivin, EGFR and bcl-2 are negative prognostic factors for patients with RCC (946-951). Thus, NR4A1 regulation of these genes in RCC contributes to the tumor phenotype, and inhibition of these genes by C-DIM/NR4A1 antagonists in RCC cells and tumors (Fig. 62) is an important component of their antineoplastic activity.

A second NR4A1-regulated pathway in cancer cells is associated with regulation of genes such as TXNDC5 and IDH1 that maintain high levels of redox equivalents and decrease intracellular stress (805). Knockdown of NR4A1 (and NR4A1 antagonists) decreases TXNDC5 and IDH1 and induced ROS and other stress responses in ACHN and 786-O cells (Fig. 63), suggesting that NR4A1 also maintains levels of stress that facilitate RCC growth and survival. The functions

of TXNDC5 and IDH1 have not been characterized in RCC and it is also possible that NR4A1 regulates other similar genes and this is currently being investigated. Since previous studies show that other ROS-inducing antineoplastic agents inhibit growth and survival of RCC (952,953), the activation of ROS by C-DIM/NR4A1 antagonists contributed to the anticancer activity of these compounds.

mTOR inhibitors are currently in clinical trials for treating RCC (932-936), and our previous studies showed that in colon and lung cancer cells expressing wild-type p53, NR4A1 inhibits p53 activity and p53-dependent inhibition of the mTOR pathway (804). However, treatment with C-DIM/NR4A1 antagonists or transfection with siNR4A1 activates p53 which in turn increases expression of sestrin 2 resulting in activation of AMPKα and inhibition of mTOR (803,805,806). These same effects were observed in ACHN cells that express wild-type p53, demonstrating that C-DIM/NR4A1 antagonists represent a novel class of mTOR inhibitors with clinical potential for treating RCC patients expressing NR4A1 and wild-type p53 (Fig. 64). Surprisingly, we also observed similar results in 786-O cells that express a mutant p53 and this response was attenuated by cotreatment with glutathione. Sestrin 2 can be induced by oxidative stress (938) and since NR4A1 knockdown and NR4A1 antagonists induce ROS (Fig. 63), this represents a second NR4A1-mediated p53-independent pathway that can be targeted by NR4A1 antagonists to inhibit mTOR.

In summary, our results show that NR4A1 is pro-oncogenic in RCC and regulates at least three pathways (Fig. 59A) important for cell proliferation and

survival, and these can be targeted by C-DIM/NR4A1 antagonists. Our current studies are focused on further investigating the functional responses and molecular pathways regulated by NR4A1 in RCC and identifying RCC patient sub-types that overexpress NR4A1 and are potential candidates for clinical applications of C-DIM/NR4A1 antagonists.

CHAPTER III

NUCLEAR RECEPTOR 4A1 (NR4A1) AS A DRUG TARGET FOR BREAST CANCER CHEMOTHERAPY*

Introduction

Nuclear receptor 4A1 (NR4A1, Nur77, TR3) is a member of the NR4A orphan receptor sub-family of nuclear receptors and the NR4A receptors (NR4A1, NR4A2 and NR4A3) play essential roles in metabolic processes, inflammation, vascular function, steroidogenesis and the central nervous system (891,892,956). NR4A1 is overexpressed in multiple tumors and cancer cell lines and results of receptor knockdown by RNA interference (RNAi) demonstrate that in solid tumors the receptor is pro-oncogenic and regulates cell growth and survival (803,805,895,899-902,937,956). Several pro-apoptotic agents including phorbol esters and adamantantyl-derived retinoids induce expression and nuclear export of NR4A1, which subsequently binds mitochondrial bcl-2 to form a pro-apoptotic complex that decreases mitochondrial membrane potential (905,938). This has led to development of peptide mimics that convert bcl-2 into an apoptotic complex and similar results have reported for the taxane-derived anticancer agent paclitaxel (906,907). Cytosporone B (CsnB) and related analogs have been

* Reprinted with permission from "Nuclear Receptor 4A1 (NR4A1) as a Drug Target for Breast Cancer Chemotherapy." Hedrick E, Lee SO, Doddapaneni R, Singh M, Safe S, 2015 Endocrine Related Cancer, 22,831-40 , Copyright[2015] Bioscientifica

identified as ligands for NR4A1 (910,911), and two additional compounds [ethyl 2-[2,3,4-trimethoxy-6-(1-oct-anoyl)phenyl]acetate (TMPA) and 1-(3,4,5-trihydroxyphenyl)no-nan-1-one (THPN)] also bind NR4A1 (941,942). TMPA inactivates nuclear NR4A1, whereas THPN and CsnB induce nuclear export of NR4A1.

Studies in this laboratory have been investigating a series of 1,1-bis(3-indolyl)-1-(*p*-substituted phenyl)methane (C-DIM) analogs and their effects on NR4A1 and NR4A1-dependent transactivation (803,805-807,896,909,937). Since NR4A1 exhibits pro-oncogenic activity, we have focused on identification of C-DIMs that inactivate NR4A1 and the *p*-hydroxyphenyl analog (DIM-C-pPhOH) was characterized as a compound that inactivated nuclear NR4A1 in cancer cell lines and this was not accompanied by nuclear export of NR4A1 (803,805,937). Subsequent studies comparing the effects of DIM-C-pPhOH and knockdown of NR4A1 (siNR4A1) by RNA interference identified three major pro-oncogenic pathways and associated genes regulated by NR4A1 that were inhibited by DIM-C-pPhOH; 1) NR4A1 regulates expression of genes such as survivin through interactions with Sp1 bound to their proximal GC-rich promoters (937); 2) NR4A1 inactivates p53 to enhance mTOR signaling in lung and colon cancer cells expressing wild-type p53 (803,806); and 3) NR4A1 regulates expression of thioredoxin domain-containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1) to maintain low levels of oxidative stress (805) (Fig. 1A).

Recent studies show that NR4A1 is overexpressed in ER-positive and ER-negative breast cancer cells (954), and NR4A1 expression in breast tumors is correlated with decreased relapse-free survival (822). Results of NR4A1 overexpression in breast cancer cells suggest that NR4A1 may be anti-migratory (955); however, a recent report indicates pro-migratory activity for this receptor (822). Research in this laboratory has demonstrated pro-oncogenic functions of NR4A1 in pancreatic, colon and lung cancer cells, and this study investigates the function of this receptor in breast cancer cells and the effects of C-DIM/NR4A1 antagonists. The results clearly demonstrate the pro-oncogenic functions of NR4A1 in breast cancer and demonstrate that C-DIM/NR4A1 antagonists represent a potential novel approach for treating patients that overexpress this orphan receptor.

Materials and Methods

Cell lines and antibodies

MCF-7, MDA-MB-231, and SKBR3 human breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic. β-Actin antibody and Dulbecco's Modified Eagle's Medium were purchased from Sigma-Aldrich (St. Louis, MO). Sp1 antibody was purchased from Millipore (Temecula, CA); caspases 7 and 8, sestrin 2 (SESN2), bcl2, CHOP, ATF4, isocitrate

dehydrogenase 1 (IDH1), and epidermal growth factor receptor (EGFR) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Caspase 3, cleaved poly ADP ribose polymerase (c-PARP; 9541), phospho mTOR, mTOR, phospho AMPKα, AMPKα, phospho p70S6K, p70S6K, phospho S6RP, S6RP, phospho 4EBP1, 4EBP1, and survivin antibodies were purchased from Cell Signaling Technologies (Danvers, MA). TXNDC5 antibody was purchased from Genetex (Irvine, CA). XBP-1s and phospho PERK were obtained from Biolegend (San Diego, CA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA). Cells were visualized under an EVOS fl, Fluorescence microscope, from Advanced Microscopy Group using a multiband filter set for FITC, rhodamine, and DAPI. The C-DIM compounds were prepared as previously described (803,805,937).

Cell proliferation assay

MCF-7, MDA-MB-231, and SKBR3 breast cancer cells (1.0×10^5 per well) were plated in 12 well plates and allowed to attach for 24 hr and cells were treated with DIM-C-pPhCO_2Me in dimethyl sulfoxide (DMSO) for 24 or 48 hr or transfected with siNR4A1 or iGL2 (control siRNA) in lipofectamine for 72 hr. Cells were then trypsinized and counted using a Coulter Z1 cell counter and growth inhibition was determined. Each experiment was carried out in triplicate, and results were expressed as the mean $\pm$ SE for each set of experiments. Cells were also treated with C-DIMs after NR4A1 knockdown.

Annexin V staining

MCF-7, MDA-MB-231, and SKBR3 cells(1.0×10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hr. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or DIM-C-pPhCO$_2$Me (15 μM) was added for 24 hr. For siRNA treatment, cells were transfected with iGL2 or 100 nm siNR4A1 (1 or 2) for 72 hr Apoptosis was analyzed by apoptotic and necrotic assay kit (Biotium CA), which contained fluorescein isothiocyanate-annexin-V, ethidium homodimer III and Hoechst 3342. Apoptosis, necrotic and healthy cell detection kit was used according to the manufacturer's protocol and cells were visualized under an EVOS fl, fluorescence microscope, from Advanced Microscopy. The proportion of apoptotic cells was determined by the amount of green fluorescence observed in the treatment groups relative and normalized to control group.

Western blot analysis

Breast cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium in six well plates. Cells were allowed to attach for 24 hr and treated with varying concentrations of DIM-C-pPhCO$_2$Me for 24 hr or with 100 nm of siNR4A1 for 72 hr. Cells were lysed with high salt lysis buffer (with protease inhibitor cocktail) and quantitated with Bradford reagent. Lysates were then analyzed by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane by wet electroblotting. Membranes were then

incubated with primary and then followed by secondary antibody. Western blot analysis was determined as described and Immobilon western chemiluminescence substrates (Millipore, Billerica, MA) were used to develop images captured on a Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR).

Small interfering RNA interference assay

Breast cancer cells were seeded (1.2 x 10^5 per well) in six well plates in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and left to attach for 24 hours. Knockdown of NR4A1 was carried out using LIpofectamine 2000 reagent according to the manufacture's protocol. Small inhibitory RNAs and GL2 (non-specific oligonucleotide) were prepared and purchased Sigma-Aldrich (St. Louis MO). The siRNA complexes used in the study are as follows: siGL2-5', CGU ACG CGG AAU ACU UCG A; siNR4A1 (1)-SASI_Hs02_00333289; siNR4A1 (2)-SASI_Hs01_00182072

Generation and measurement of ROS

Cellular ROS levels were ascertained using the cell permeable probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen (Grand Island, NY). Following treatment of the cells for 12 or 24 hr with DIM-C-pPHCO_2Me or siNR4A1 for 72 hr, cells were plated on a 6-well culture plate were trypsinized, neutralized, then loaded with 10 μM of probe for 20 min, washed once with serum free medium, and then ROS was

measured by flow cytometry using Accuri's C6 Flow Cytometer (Accuri, Ann Arbor, MI).

TNBC orthotopic xenograft model

Female BALB/c nude mice (6-8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA) and maintained under specific pathogen-free conditions, housed in isolated vented cages and allowed to acclimate for one week with standard chow diet. The animals were housed at Florida A&M University in accordance with the standards of the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The protocol of the animal study was approved by the Institutional Animal Care and Use Committee (IACUC), Florida A&M University, Fl. MDA-MB-231 cells ($1X10^6$ cells) were detached, resuspended in 100 μl of phosphate-buffered saline with matrigel (BD Bioscience, Bedford, MA), and implanted subcutaneously in the mammary fat pad of mice. When tumors reached about 40-500 mm^3 size, the animals were randomized into control and treatment groups (6 animals per group) and mice were treated with placebo or DIM-C-pPhCO_2Me or DIM-C-pPhCN (50 mg/kg/d) in nano liquid carrier (administered in sodium carboxymethyl cellulose) by oral gavage every second day for 4 weeks. Tumor volumes and weights, and body weight were determined; the tumor size was measured using Vernier calipers, and the tumor volume was estimated by the formula: tumor volume (mm^3) = (L X W^2) x ½, where L is the

length and W is the width of the tumor. Tumor lysates were obtained and analyzed for protein expression by western blots.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by Student's *t* test. The results are expressed as means with error bars representing 95% confidence intervals for 3 experiments for each group unless otherwise indicated, and a *P* value less than 0.05 was considered statistically significant. All Statistical tests were 2-sided.

Results

Figure 66.

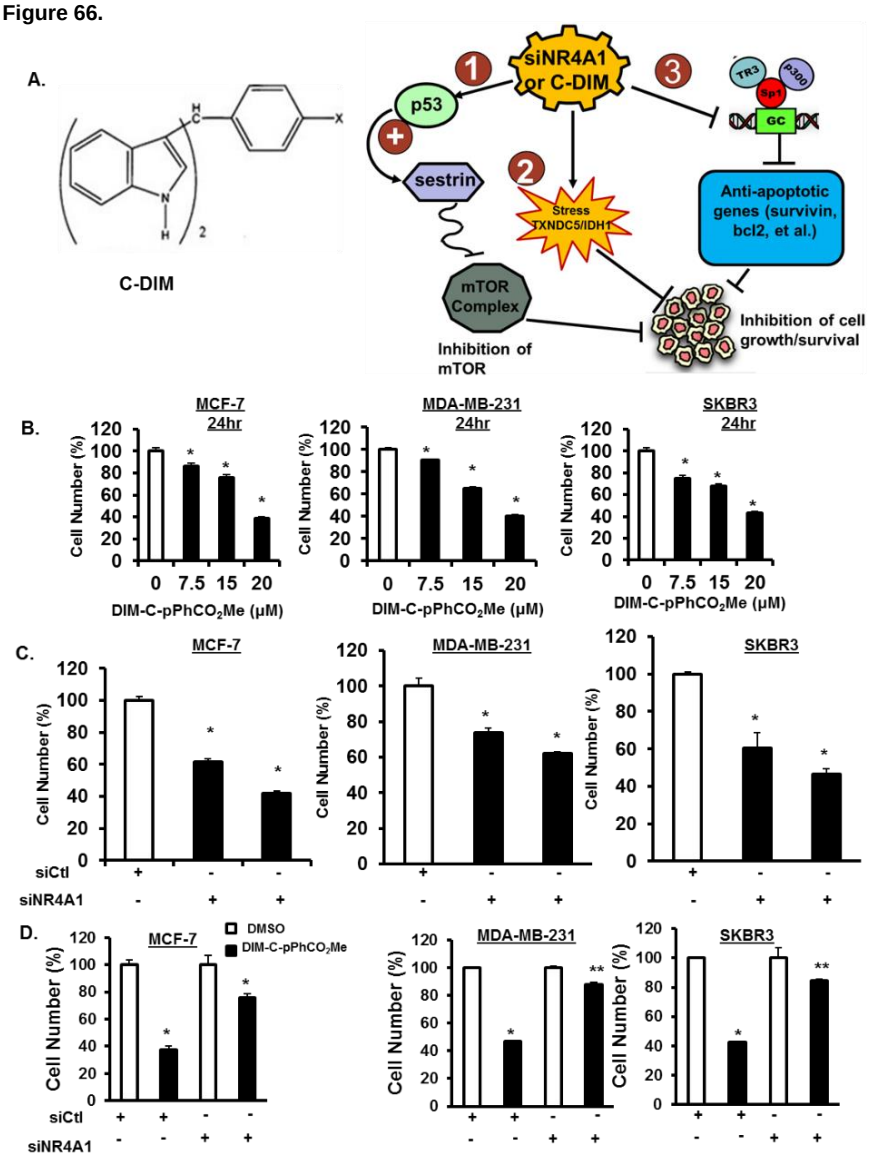

Figure 66. NR4A1-regulated pathways and effects of NR4A1 knockdown on breast cancer cell proliferation. (A) NR4A1-regulated pathways/genes that can be targeted by C-DIM/NR4A1 antagonists. (B) Cells were transfected with two siNR4A1 oligonucleotides (1 and 2) and cell numbers were determined after 72 hr. (C) Cells were treated with different concentrations of DIM-C-pPhCO$_2$Me for 24 hr and the number of cells were then determined. (D) Cells were transfected with siCtl (non-specific oligonucleotide) or siNR4A1 and then treated with 20 μM DIM-C-pPhcO$_2$Me for 24 hr and the number of cells were then counted. Results (B – D) are means ± SE for at least 3 separate determinations for each treatment group. Significant ($p < 0.05$) growth inhibition is indicated (*) and a significant decrease in the growth inhibitory effects of DIM-C-pPhCO$_2$Me after NR4A1 knockdown is also indicated (**).

Inhibition of cell proliferation by siNR4A1 and DIM-C-pPhCO$_2$Me

The orphan nuclear receptor NR4A1 is overexpressed in ER-positive and ER-negative breast cancer cells and the role of this receptor in regulating breast cancer cell growth and survival was investigated by RNA interference (RNAi) in ER-positive (MCF-7), ER-negative (MDA-MB-231) and erbB2 (SKBR3) overexpressing breast cancer cell lines. Figure 66A summarizes the pathways regulated by NR4A1 and the structure of our C-DIM/NR4A1 anatgonists. Cells were transfected with two different oligonucleotides against NR4A1 (siNR4A1-1/siNR4A1-2) and this resulted in ≥50% growth inhibition in MCF-7 and SKBR3 cells and 35% inhibition of MDA-MB-231 cell proliferation (Fig. 66B). The C-DIM compound with a *p*-carboxymethylpenyl group (DIM-C-pPhCO$_2$Me) has been identified as an NR4A1 antagonist, which inhibits nuclear NR4A1 (Suppl. Fig. A-3) and Figures 66C and this compound significantly inhibits growth of MCF-7, MDAMB-231 cells after treatment for 24 and 48 hr (Suppl. Fig. A-4A). IC_{50} values were 19.74, 18.62 and 18.92 μM after treatment of MCF-7, MDA-MB-231 and SKBR3 cells for 24 hr and 12.73,19.02 and 12.28 μM after treatment for 48 hr. In contrast, after knockdown of NR4A1 in these cells, treatment with DIM-C-pPhCO$_2$Me resulted in only minimal growth inhibition confirming a role for NR4A1 in mediating the growth inhibitory effects of DIM-C-pPhCO$_2$Me (Fig. 66D).

Figure 67.

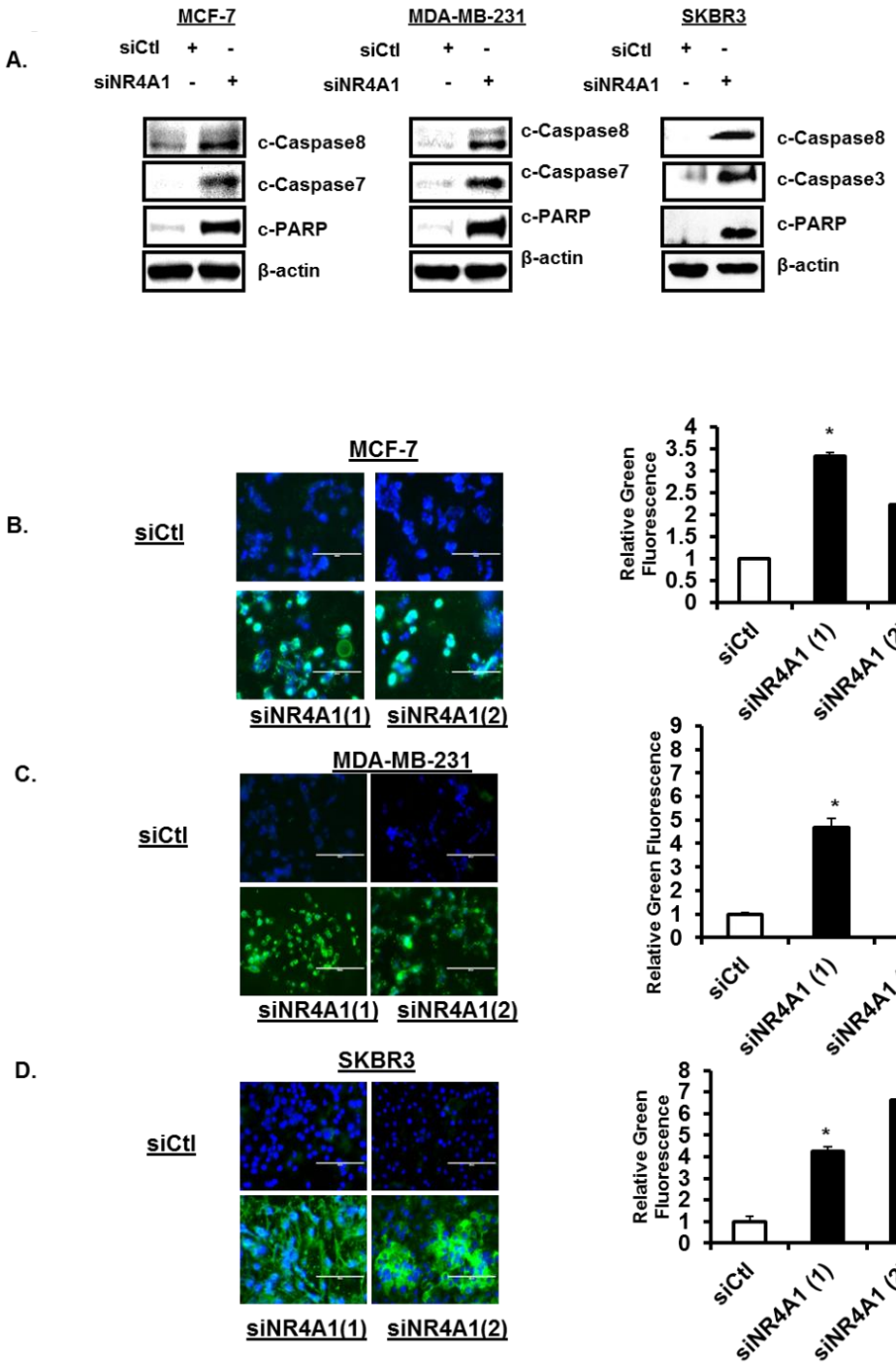

Figure 67. siNR4A1 induces apoptosis in breast cancer cells. (A) Cells were transfected with siNR4A1 and whole cell lysates were analyzed by western blots. Cells were transfected with siNR4A1 (2 oligonucleotides) and effects on Annexin V staining in MCF-7 (B), MDA-MB-231 (C) and SKBR3 (D) cells were determined and quantitated. Results (B – D) are means ± SE for at least 3 separate determinations and significant ($p < 0.05$) induction of Annexin V staining is indicated (*).

siNR4A1 and DIM-C-pPhCO$_2$Me induce apoptosis in breast cancer cells

NR4A1 also regulates pro-survival genes and pathways in pancreatic and lung cancer cells, and results in Figure 67A show that transfection of breast cancer cells with siNR4A1 induced cleavage of caspase 8 and caspase 7 and also PARP cleavage. Moreover, siNR4A1 also induced annexin V staining MCF-7 (Fig. 67B), MDA-MB-231 (Fig. 67C), and SKBR3 (Fig. 67D) cells confirming that NR4A1 regulated anti-apoptotic pathways in these cell lines. Treatment of the cells with DIM-C-pPhCO$_2$Me for 24 hr also induced cleavage (activation) of caspases 7 and 8 and PARP (Fig. 68A) and similar results were observed for the *p*-cyanophenyl compound (DIM-C-pPhCN) (Suppl. Fig. A-4B)which is an NR4A1 ligand and antagonist in colon cancer cells (806). DIM-C-pPhCO$_2$Me also enhanced annexin V staining in MCF-7 (Fig. 68B), MDA-MB-231 (Fig. 68C), and SKBR3 (Fig. 68D) cell lines. Thus, both NR4A1 knockdown and NR4A1 antagonists decreased breast cancer cell growth and induced apoptosis.

Figure 68.

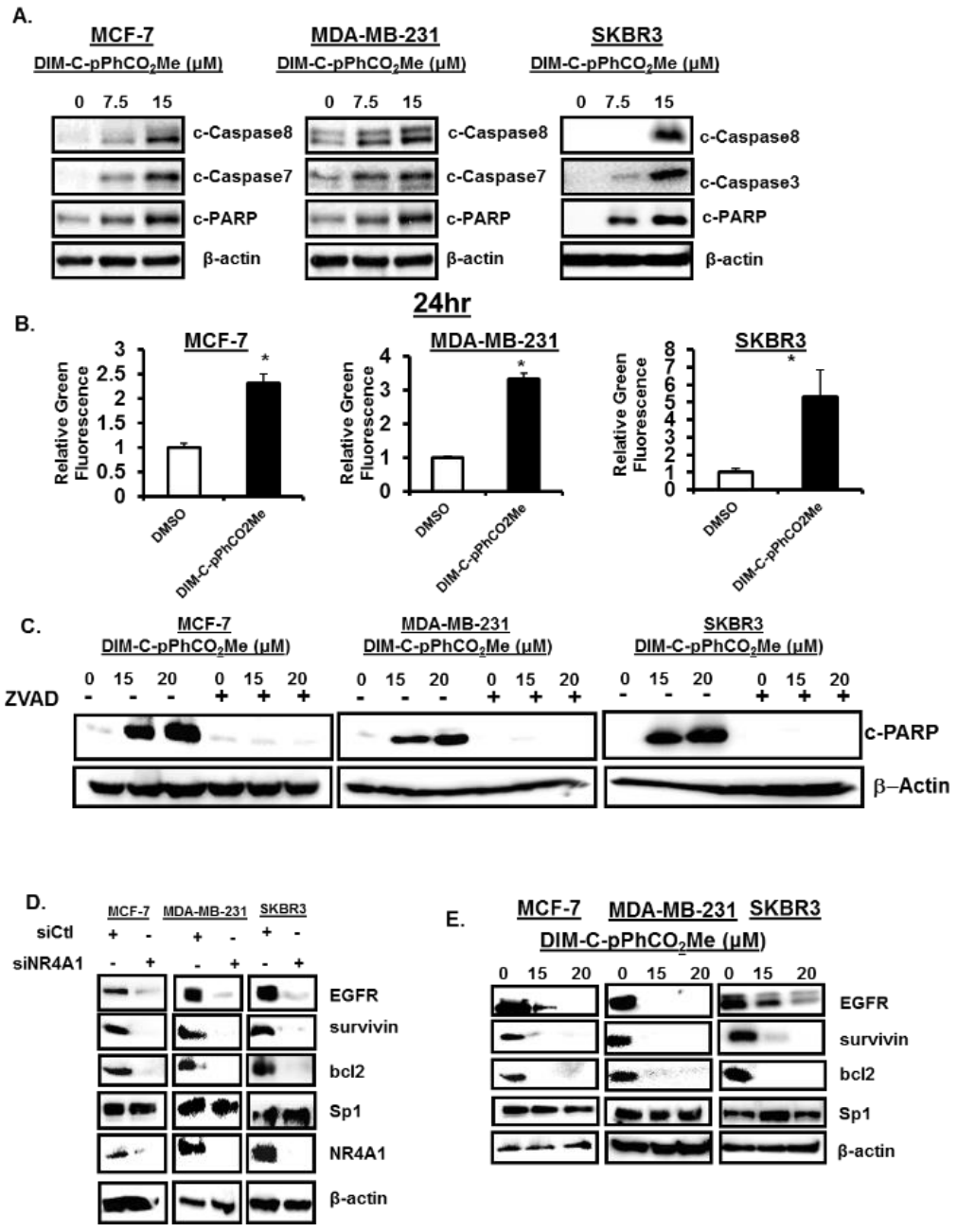

Figure 68. DIM-C-pPhCO$_2$Me induces apoptosis in breast cancer cells. (A) Cells were treated with DIM-C-pPhCO$_2$Me for 24 hr and whole cell lysates were analyzed by western blots. MCF-7, MDA-MB-231,and SKBR3 cells were treated with DIM-C-pPhCO$_2$Me for 24 hr and Annexin V staining was determined and quantitated (B). DIM-C-pPhCO$_2$Me also induced PARP cleavage (C). siNR4A1 (D) and DIM-C-pPhCO$_2$Me (E) decreased Sp regulated gene expression without decreasing Sp1 expression. Quantitative results are means ± SE for 3 separate determinations and significant ($p < 0.05$) induction of Annexin V is indicated (*).

siNR4A1 and DIM-C-pPhCO$_2$Me activate growth inhibitory pro-apoptotic pathways/genes in breast cancer cells

mTOR activation in lung and colon cancer cells is dependent on NR4A1-p53 interactions that inactivate p53, and siNR4A1 or NR4A1 antagonists activate p53 which induces expression of sestrin 2, resulting in phosphorylation of AMPKα and inhibition of mTOR (803,806). Knockdown of NR4A1 in p53-wild-type MCF-7 cells resulted in the induction of sestrin 2 and phosphorylation of AMPKα and this was accompanied by decreased activation (phosphorylation) of the mTOR downstream gene products p70S6K and S6RP (Fig. 69A). MCF-7 cells were also treated with the NR4A1 antagonist DIM-C-pPhCO$_2$Me (Fig. 69B) and DIM-C-pPhCN (Suppl. Fig. A-4C) and results were similar to that observed after transfection with siNR4A1. Previous studies also show that NR4A1 regulates expression of growth promoting and pro-survival (e.g. survivin, bcl-2 and EGFR) genes through interactions with Sp1 bound to their corresponding proximal GC-rich *cis*-promoter elements (803,806,937). Figure 69C shows that after knockdown of NR4A1 (siNR4A1) in MCF-7, MDA-MB-231 and SKBR3 cells, there was a significant decrease in expression of several Sp1-regulated genes including EGFR, survivin and bcl-2; however, Sp1 protein levels were unchanged. Similar results were observed in the same cell lines after treatment with the NR4A1 antagonists DIM-C-pPhCO$_2$Me (Fig. 69D) and DIM-C-pPhCN (Suppl. Fig.A-4D). These observations are consistent with previous reports in pancreatic, colon and

lung cancer cells where NR4A1 regulates expression of survivin and other Sp1-regulated genes (803,806,937).

Figure 69.

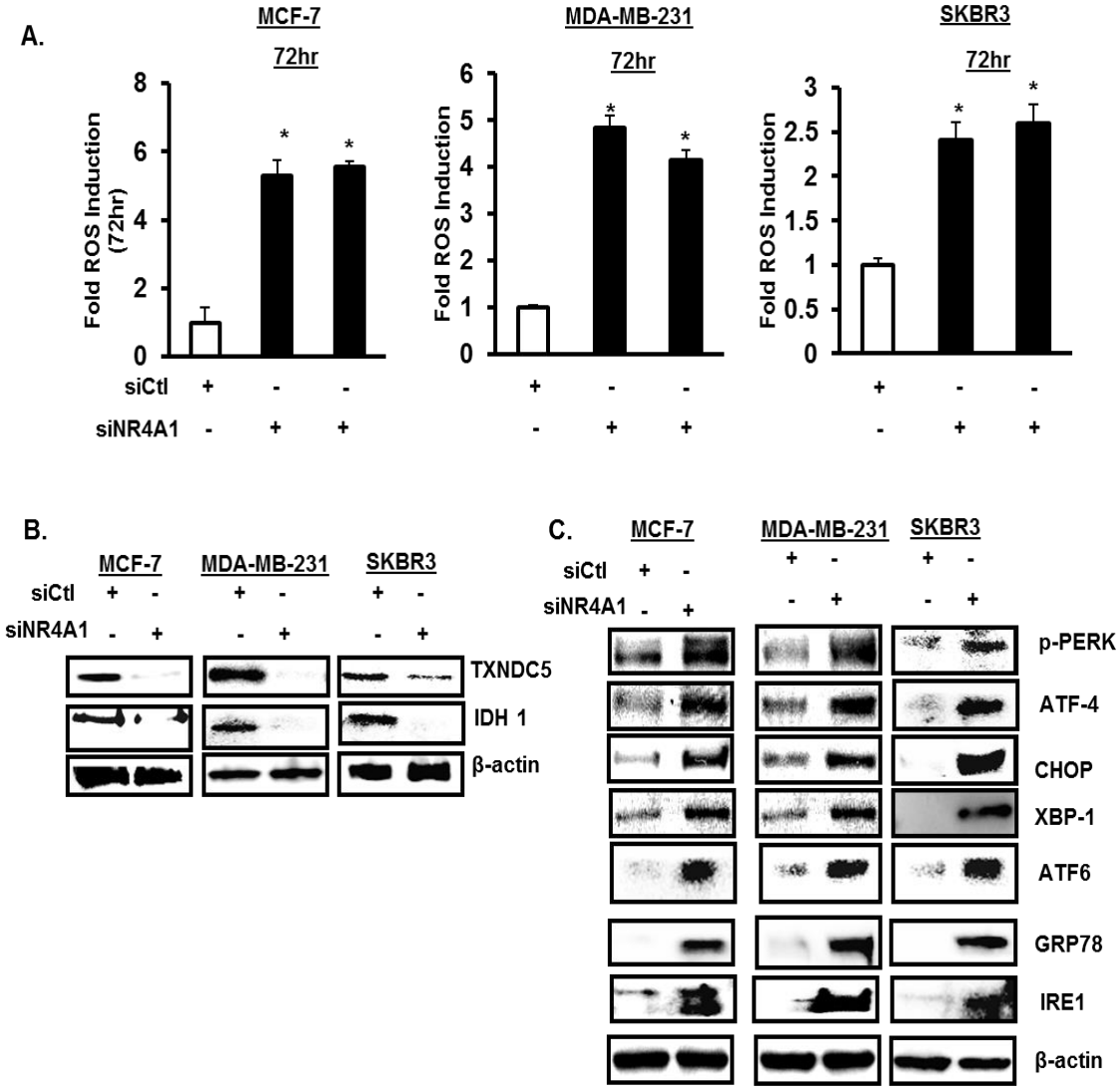

Figure 69. NR4A1 knockdown induces ROS and ER stress. (A) Cells were transfected with siNR4A1 or siCtl and ROS was determined. (B) Cells were transfected with siNR4A1 and whole cell lysates were analyzed by western blots for IDH1 or TXNDC5 and ER stress gene products (C). Results in (A) are means ± SE for 3 separate determinations and significant ($p < 0.05$) induction of ROS is indicated (*).

It was recently reported that NR4A1 regulates expression of genes such as IDH1 and TXNDC5 that maintain high levels of reducing equivalents and minimize ROS-mediated cellular stress (805,806). Knockdown of NR4A1 by RNAi induced ROS by 2- to 4-fold in MCF-7, MDA-MB-231 and SKBR3 cells and this was accompanied by decreased expression of both TXNDC5 and IDH1 in these cell lines (Fig. 70B). Moreover, after transfection with siNR4A1, we also observed enhanced markers of ER stress including increased phosphorylation of PERK and increased expression of ATF-4, CHOP and XBP-1 in the breast cancer cell lines (Fig. 70C). Treatment of MCF-7, MDA-MB-231 and SKBR3 cells with the NR4A1 antagonist DIM-C-pPhCO$_2$Me also increased ROS after 12 and 24 (Figs. 71A-71C) hr and this was also accompanied by decreased expression of TXNDC5 and IDH1 and induction of markers of ER stress (p-PERK, ATF4, CHOP and XBP-1) as previously observed in pancreatic cancer cells (805). We also observed that the NR4A1 antagonist DIM-C-pPhCN decreased expression of IDH1 and TXNDC5 in breast cancer cells (Suppl. Fig. A-4E).

Figure 70.

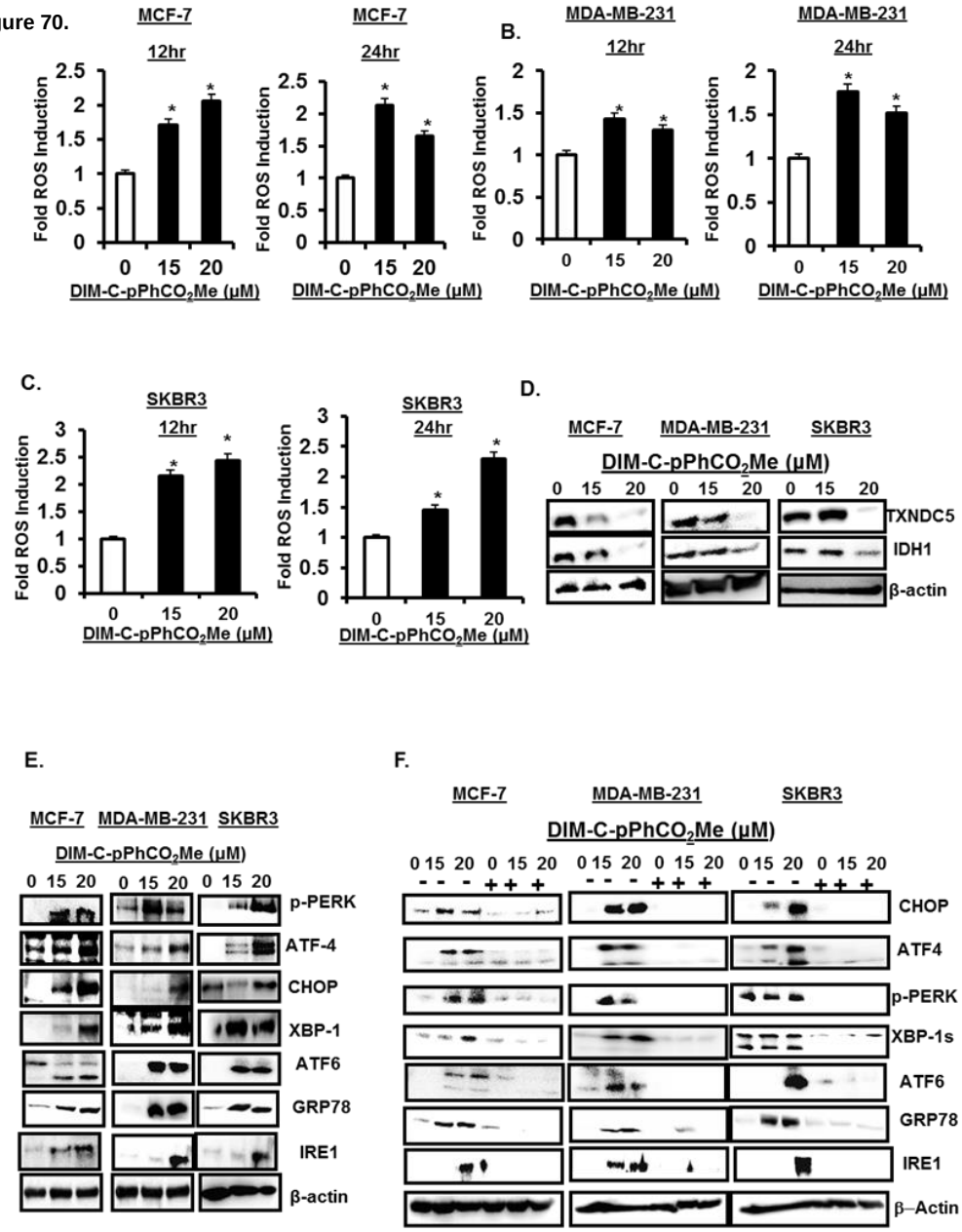

Figure 70. DIM-C-pPhCO$_2$Me induces ROS and ER stress. MCF-7 (A), MDA-MB-231 (B) and SKBR3 (C) cells were treated with DIM-C-pPhCO$_2$Me and ROS was determined after 12 or 24 hr. (D) Cells were treated with DIM-C-pPhCO$_2$Me and whole cell lysates were analyzed by western blots for expression of TXNDC5, IDH1 and stress gene products (E). (F) Cells were treated with DMSO, DIM-C-pPhCO2Me alone or in combination with GSH, and whole cell lysates were analyzed for stress gene products by western blots. Results (A) are expressed as means ± SE for 3 separate determinations and significant ($p < 0.05$) induction of ROS is indicated (*).

Figure 71.

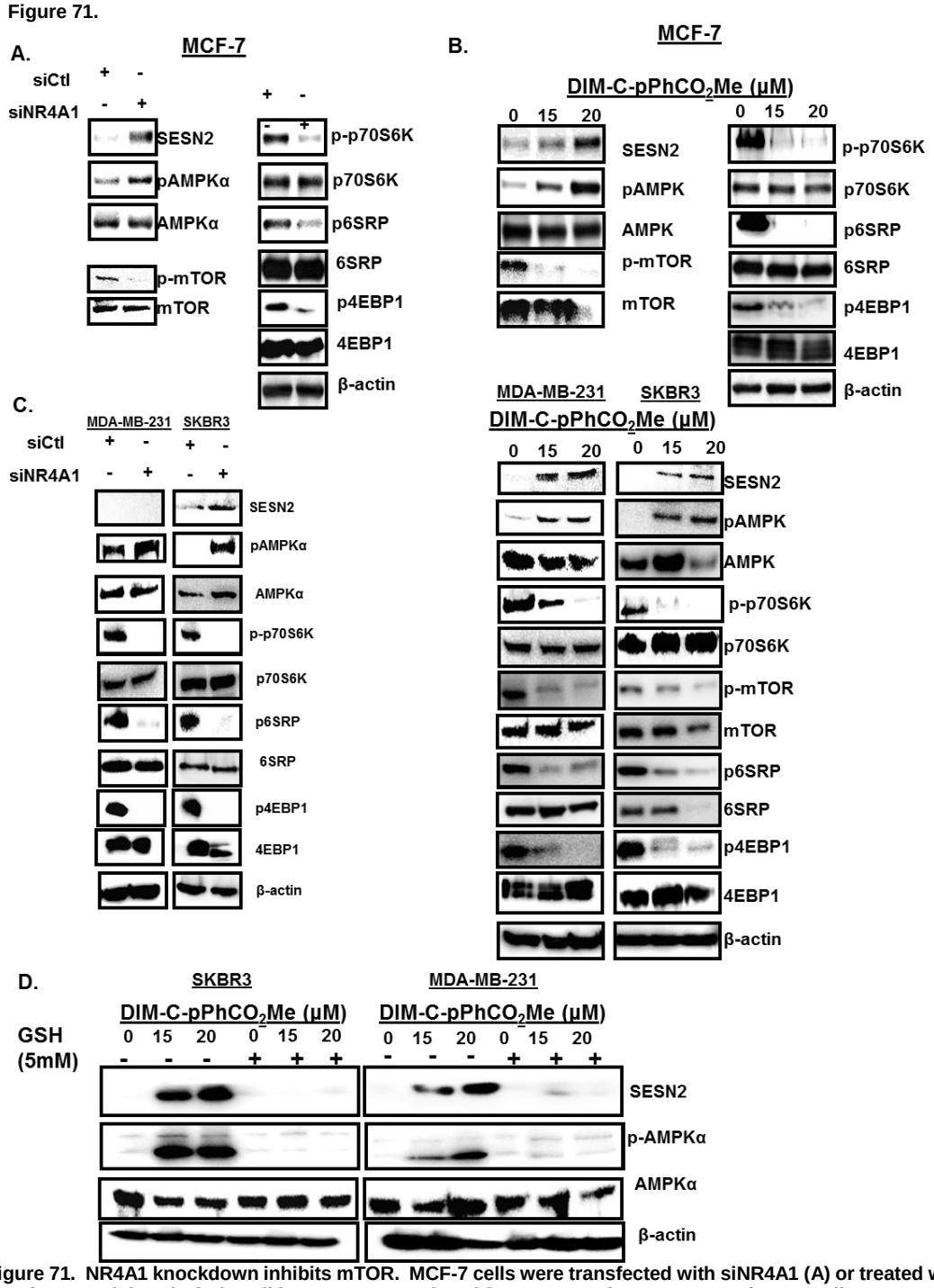

Figure 71. NR4A1 knockdown inhibits mTOR. MCF-7 cells were transfected with siNR4A1 (A) or treated with DIM-C-pPhCO$_2$Me (B) and whole cell lysates were analyzed for mTOR pathway gene products. Cells were transfected with siNR4A1 (C) or treated with DIM-C-pPhCO$_2$Me (D) and whole cell lysates were analyzed by western blots for selected genes with GC-rich promoters.

The effects of DIM-C-pPhCO$_2$Me and DIM-C-pPhCN as inhibitors of mammary tumorigenesis were investigated in an orthotopic model using MDA-MB-231 cells in athymic nude mice. At a dose of 50 mg/kg/d, DIM-C-pPhCO$_2$Me inhibited tumor growth and weight (Fig. 72A). Western blot analysis of tumor lysate from control and DIM-C-pPhCO$_2$Me-treated mice confirmed that DIM-C-pPhCO$_2$Me significantly induced PARP cleavage, decreased expression of Sp-regulated survivin, EGFR and bcl2 gene products and also decreased levels of TXNDC5 and IDH1 (Figs. 72B and 72C). This effect was also observed for DIM-C-pPhCN (These results are consistent with the *in vitro* effects of DIM-C-pPhCO$_2$Me (and siNR4A1) in breast cancer cells and demonstrates that C-DIM/NR4A1 antagonists inhibit common NR4A1-mediated pro-oncogenic pathways in breast, pancreatic, colon and lung cancer cell lines.

Figure 72.

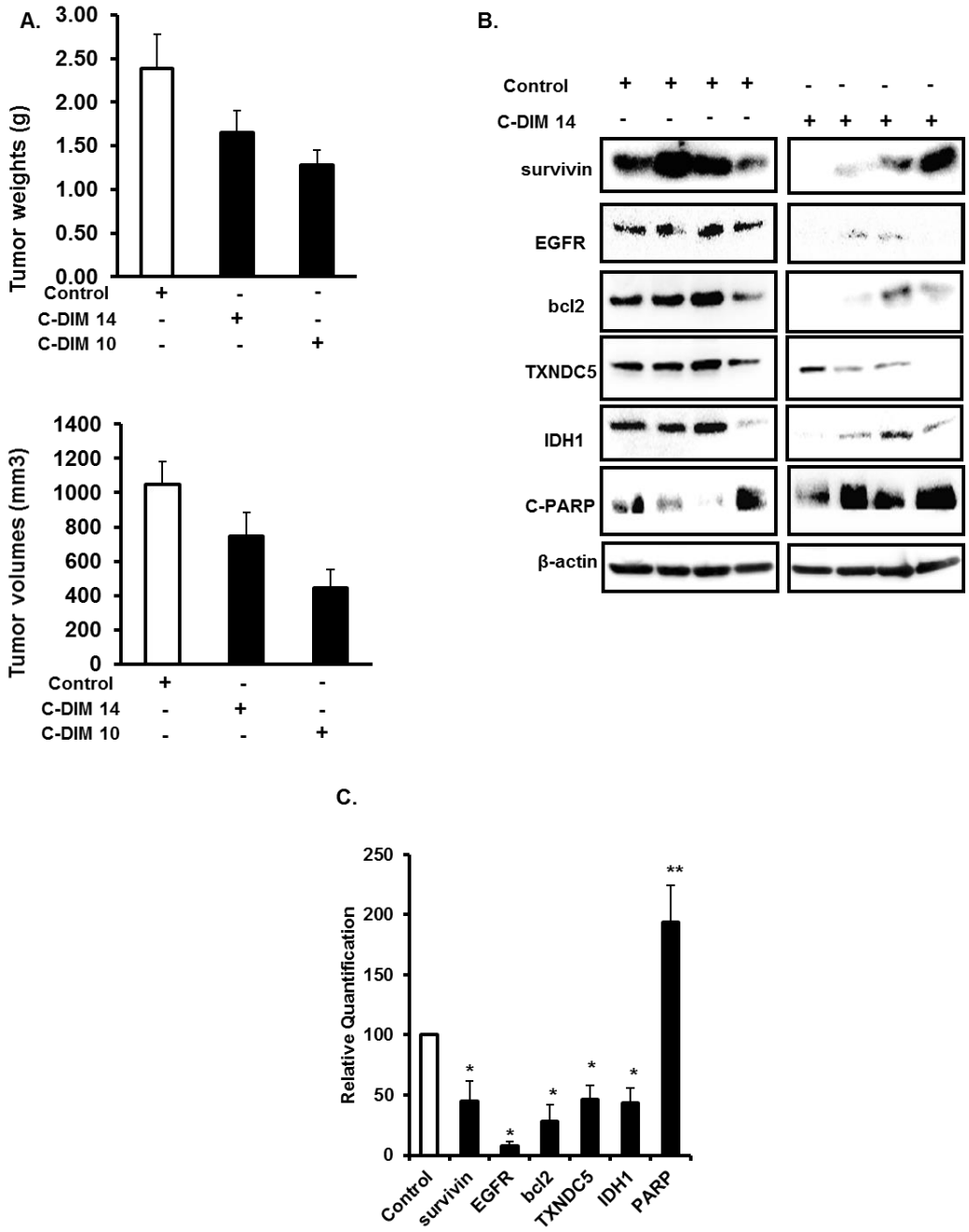

Figure 72. Inhibition of tumor growth by NR4A1 antagonists. (A) Orthotopic tumors in athymic nude mice bearing MDA-MB-231 cells were treated with 50 mg/kg/d DIM-C-pPhCO$_2$Me or DIM-C-pPhCN o corn oil (control) for 28 days and tumor volumes were determined. (B) Lysates from tumors from control and DIM-C-pPhCO$_2$Me-treated mice were analyzed by western blots and bands were quantitated (C) and normalized relative to β-actin.

Discussion

NR4A1 exhibits pro-oncogenic activity in cancer cell lines derived from solid tumors and is overexpressed in tumors from patients with lung, pancreatic and colon tumors (803,805-807,895,896,937). Moreover, in lung cancer patients, high expression of NR4A1 is a negative prognostic indicator for decreased survival (803). Similar results were recently reported for the expression and prognostic activity of NR4A1 in breast cancer patients, and it was also observed that NR4A1 was one of only a few nuclear receptors overexpressed in both ER-positive and ER-negative breast tumors (822,954). Since an early report indicated that NR4A1 is more highly expressed in early vs. late stage more aggressive breast tumors and exhibited some tumor suppressor-like activity (955), we investigated the function of NR4A1 in three different breast cancer cell lines by RNA interference and treatment with NR4A1 antagonists. We have recently identified several C-DIMs including the *para*-hydroxy, carbomethoxy, cyano and bromophenyl analogs that directly bind the ligand binding domain of NR4A1 and exhibit NR4A1 antagonist activity in colon cancer cells (806). The *p*-carbomethyoxyphenyl analog (DIM-C-pPhCO_2Me) was used as a prototypical C-DIM/NR4A1 antagonist for investigating the anticancer activity drugs targeting NR4A1 in breast cancer cells and we have confirmed that DIM-C-pPhCN also exhibits similar activities.

Initial studies investigated the role of NR4A1 in the growth of three prototypical breast cancer cell lines (ER-positive MCF-7, erbB2 overexpression

SKBR3, and triple negative MDA-MB-231), and knockdown of NR4A1 using two different oligonucleotides significantly decreased proliferation of all three breast cancer cell lines and similar growth inhibitory effects were observed for DIM-C-pPhCO$_2$Me (Fig. 1A-1C). Moreover, after knockdown of NR4A1 in MCF-7, MDA-MB-231 and SKBR3 cells, treatment with DIM-C-pPhCO$_2$Me had minimal effects (Fig. 66), suggesting that the growth inhibitory effects of DIM-C-pPhCO$_2$Me were primarily NR4A1-dependent (Fig. 66D). The effects of NR4A1 knockdown and treatment with DIM-C-pPhCO$_2$Me on several markers of apoptosis, including cleavage of caspases 7 and 8 and PARP and induction of annexin V staining, were also determined in the breast cancer cell lines (Figs. 67 and 68). These results demonstrate that NR4A1 regulates pathways that contribute to the growth and survival of breast cancer cells and this parallels the functions previously observed for this receptor in pancreatic, lung and colon cancer cells (803,805,806,937).

NR4A1 binds and inactivates p53 (939), and knockdown of NR4A1 or treatment of p53 wild-type lung cancer cells with an NR4A1 antagonist or transfection with siNR4A1 results in activation of p53 (803). p53 induces sestrin 2 which activates AMPKα and this leads to inhibition of the mTOR pathway (803). The mTOR pathway inhibitors have been extensively developed for cancer chemotherapy (955,956), and C-DIM/NR4A1 antagonists are a new class of mTOR inhibitors which block NR4A1-regulated mTOR activation (803). Results illustrated in Figures 4A and 4B show that both siNR4A1 and DIM-C-pPhCO$_2$Me

inhibited mTOR pathway in MCF-7 breast cancer cells that express wild-type p53. In p53 wild-type lung cancer cells, siNR4A1 and NR4A1 antagonists also induce sestrin 2 which activates AMPKα and inhibits mTOR, whereas this is not observed in lung cancer cells expressing mutant p53 (803). Interestingly, DIM-C-pPhCO_2Me and siNR4A1 induced sestrin 2 and inhibited mTOR in p53 mutant SKBR3 and MDA-MB-231 cells (data not shown) and the mechanisms of this response are currently being investigated. Like other nuclear receptors, NR4A1 interacts with the Sp1 transcription factor bound to GC-rich sites to activate survivin and other anti-apoptotic/growth promoting genes, and siNR4A1 or treatment with a NR4A1 antagonist decreases expression of these genes (957-962) (Fig. 72D). Figures 69C and 69D show that siNR4A1 or treatment with DIM-C-pPhCO_2Me decreased expression of survivin, bcl2 and EGFR in MDA-MB-231, MCF-7 and SKBR3 cells; however, Sp protein levels were unchanged. Molecular analysis of NR4A1-dependent regulation of survivin showed that in pancreatic cancer cells, NR4A1 and p300 cooperatively activated survivin expression by interacting with Sp1 bound to the proximal GC-rich region of the survivin promoter (937). Regulation of growth-promoting and survival genes which contain GC-rich promoters by NR4A1 is consistent with the growth inhibitory and apoptotic effects of siNR4A1 and C-DIM/NR4A1 antagonists on breast cancer cells (Fig. 1) and tumors (Figs. 72A-72C) and is comparable to that observed in lung, colon and pancreatic cancer cell lines (803,805,806,937).

A recent study showed that NR4A1 maintains low levels of oxidative and endoplasmic reticulum (ER) stress in pancreatic cancer cells by regulating expression of TXNDC5 and IDH1 which maintain cellular levels of reducing equivalents (805). DIM-C-pPhCO_2Me or siNR4A1 also decreased expression of TXNDC5 and IDH1 in breast tumors (*in vivo*) (Figs. 72B-72C) and MCF-7, MDA-MB-231 and SKBR3 cells, and this was accompanied by increased levels of ROS and induction of markers of ER stress (Figs. 70 and 71). These results are consistent with previous studies in pancreatic cancer cells, and there is also emerging evidence that both TXNDC5 and IDH1 are overexpressed in breast cancer cells and tumors (963,964) and their expression and functions in breast cancer cells are currently being investigated.

In summary, results of this study are consistent with a pro-oncogenic role for NR4A1 in breast cancer as an important regulator of cell growth and survival, and NR4A1-regulated pro-oncogenic pathways and genes are similar to those observed in pancreatic, lung and colon cancer cells and tumors (803,805,806,937). This study also demonstrated the effectiveness of the NR4A1 antagonist DIM-C-pPhCO_2Me as an inhibitor of breast cancer growth and survival and current structure-activity studies are focused on identifying the most effective C-DIM/NR4A1 antagonists for future clinical applications.

CHAPTER IV

HISTONE DEACETYLASE INHIBITORS INHIBIT RHABDOMYOSARCOMA BY REACTIVE OXYGEN SPECIES TARGETING OF SPECIFICITY PROTEIN TRANSCRIPTION FACTORS*

Introduction

Rhabdomyosarcomas (RMS) is the most common soft tissue sarcoma that is primarily observed in children and adolescents and accounts for 5% of all pediatric cancers and 50% of soft tissue sarcomas in children (965-967). Embryonal RMS (ERMS) and alveolar RMS (ARMS) are the two major classes of RMS in children and adolescents and differ with respect to their histology, genetics, treatment, and prognosis (968,969). ERMS accounts for over 60% of RMS patients and is associated with loss of heterozygosity at the 11p15 locus (970). ERMS patients have a favorable initial prognosis; however, the overall survival of patients with metastatic ERMS is only 40% (971). ARMS occurs in approximately 20% of RMS patients and is associated with translocations resulting in formation of pro-oncogenic gene products resulting from the fusion of PAX3 or PAX7 with the Forkhead gene FOXO1A (972,973). ARMS patients have

*Reprinted with permission from "Histone Deacetylase Inhibitors Inhibit Rhabdomyosarcoma by Reactive Oxygen Species-Dependent Targeting of Specificity Protein Transcription Factors." Hedrick E, Crose L, Linardic CM, Safe S, 2015 Molecular Cancer Therapeutics, 14,2143-53,Copyright[2015] American Association for Cancer Research

a poor diagnosis and patient survival is <20% for metastatic ARMS. Treatments include radiotherapy, surgery, and chemotherapy with cytotoxic drugs and/or drug combinations of vincristine, dactinomycin, cyclophosphamide, irinotecan, fosfamide, etoposide, doxorubicin and others (974,975). A serious problem also exists for RMS patients that survive current therapies since these individuals as adults have an increased risk for several diseases (626). Thus, there is a critical need for development of new therapeutic regimens for treating childhood RMS. Specificity protein 1 (Sp1) transcription factor is overexpressed in human RMS tumors and other Sp family members including Sp3 and Sp4 are also overexpressed in RMS cell lines (976). The importance of Sp transcription factors (TFs) in RMS is primarily due to pro-oncogenic Sp-regulated genes that are themselves drug targets for RMS and these include CXCR4, hepatocyte growth factor receptor (C-MET), insulin-like growth factor 1 receptor (IGF-1R), and platelet-derived growth-factor receptor α (PDGFRα) (977-980). Clinical studies using drugs that specifically target Sp TFs and Sp-regulated genes for treatment of RMS have not yet been reported; however, there is an open phase I/II trial (NCT01610570) evaluating the efficacy of mithramcyin in solid tumors including RMS. Mithramycin acts in part by binding to GC-rich sequences and regulating chromatin accessibility, including the ability to displace Sp1 from oncogenic promoters. Thus the therapeutic potential of Sp TF in RMS is gaining traction.

Genomic analysis of RMS from several patients indicated that “skeletal muscle (rhabdomyosarcoma) may have even higher levels of ROS than other

cancer cells and may be particularly sensitive to therapeutics that induce oxidative stress (650). This sensitivity is thought to occur because with such a high baseline burden of ROS, there is little tolerance for further oxidative stress and this was confirmed by showing that ROS inducers were highly effective inhibitors of RMS tumor growth using patient-derived xenografts in mouse models (650). Recent studies in our laboratory (648) demonstrate that ROS inducers also inhibit pancreatic cancer cell and tumor growth and this is due, in part, to a novel epigenetic pathway (781) in which ROS-mediated repression of c-Myc results in downregulation of Sp TFs and pro-oncogenic Sp-regulated genes. In this study, we demonstrate that ROS-inducing histone deacetylase (HDAC) inhibitors block RMS cell and tumor growth by initially targeting c-Myc which results in downregulation of microRNAs (miRs) and induction of ZBTB transcriptional repressors which in turn downregulate Sp TFs.

Materials and Methods

Cell lines and antibodies

RD and Rh30 rhabdomyosarcoma cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained 37˚C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic or RPMI-1640 Medium with 10% fetal bovine serum and antibiotic respectively. B-actin antibody Dulbecco's

Modified Eagle's Medium, and RPMI-1640 Medium, and 36% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Hematoxylin was purchased from Vector Laboratories (Burlingame, CA). Sp1 antibody and Glutathione (GSH) reduced free acid were purchased from Millipore (Temecula, CA); Sp3, Sp4 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Acetyl-Histone H3 (K9/K14) and c-Myc antibodies were purchased from Cell Signaling technologies (Danvers, MA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA). Cells were visualized under an EVOS fl, Fluorescence microscope, from Advanced Microscopy Group using a multiband filter set for FITC, rhodamine, and DAPI. The panobinostat and vorinostat were purchased from LC laboratories (Woburn, MA).

Cell proliferation assay

RD and Rh30 rhabdomyosarcoma cells (1.0×10^5 per well) were plated in 12 well plates and allowed to attach for 24 hours and cells were treated with panobinostat and vorinostat (dimethyl sulfoxide, DMSO, as empty vehicle) (+/- GSH 3 h prior to treatment) for 24 hours or by siSp1, siSp3, siSp4 (iGL2 as control siRNA with lipofectamine vehicle) for 72 hours. Cells were then trypsinized and counted after respective treatment time intervals using a Coulter Z1 cell counter and growth inhibition was determined. Each experiment was carried out in triplicate, and results were expressed as the mean ± SE for each set of experiments.

MTT assay

Primary human myoblasts (HSMM, Lonza), Rh30, or RD cells were plated in 96-well plates at a density of 10,000 cells per well. The next day, cells were treated with vehicle (DMSO) or increasing doses of Panobinostat. Twenty-four hours post-treatment, cell viability was analyzed by MTT assay, as described previously (Crose et al, Clin Cancer Research 2012).

Annexin V staining

RD and Rh30 rhabdomyosarcoma cell (1.0×10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO Panobinostat (50-100 nM) or Vorinostat (0.5-1.0 µM) (+/-5mM GSH 3 h prior to treatment) was added for 24 hr. For siRNA treatment, cells were treated with iGL2 or 100 nm siSp1, siSp3, siSp4 siRNA for 72 hours. Apoptosis was analyzed by apoptotic and necrotic assay kit (Biotium CA), which contained fluorescein isothiocyanate-annexin-V, ethidium homodimer III and Hoechst 3342. Apoptosis, necrotic and healthy cell detection kit was used according to the manufacturer's protocol and were visualized under an EVOS fl, fluorescence microscope, from Advanced Microscopy. The proportion of

apoptotic cells was determined by the amount of green fluorescence observed in the treatment groups relative and normalized to control group.

Boyden Chamber Assay

RD and Rh30 rhabdomyosarcoma cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. Cells were seeded and subsequently treated with varying concentration of panobinostat or vorinostat for 24 hours (+/- GSH 3 h prior to treatment) or with 100 nm of siSp1, siSp3, siSp4 for 48 hours. Cells were trypsinized, counted then placed in 12-well 8.0µm pore ThinCerts from Greiner bio-one (Monroe , NC) allowed to migrate for 24 hr, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted.

RT-PCR

miRNA was isolated using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. Quantification of miRNA (RNU6B and miR-17, -20a, and -27a) was done using the TaqMan miRNA assay kit (Life Technologies) according to the manufacturer's protocol with real-time PCR. U6 small nuclear RNA was used as a control to determine relative miRNA expression.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. RD and Rh30 cells were treated with 100 nM panobiostat for 3 or 6 hr. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (~200 to 1,500 bp). The sonicated chromatin was immunoprecipitated with normal IgG (Santa Cruz), H3K27me3 (Abcam), H3K4me3 (Abcam), H4K16Ac (Active Motif), or RNA polymerase II (pol II; GeneTex) antibodies and protein A-conjugated magnetic beads at 4°C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. DNA was prepared by proteinase K digestion followed by PCR amplification. The primers for detection of the c-Myc promoter region were 5′-GCC CTT TCC CCA GCC TTA GC-3′ (sense) and 5′-AAC CGC ATC CTT GTC CTG TGA GTA-3′ (antisense), the primers for detection of the Sp1 promoter region were 5′-CTA ACT CCA ATC ATA ACG TTC C-3′ (sense) and 5′-GAG CTG GAG ATG ATT GGC TTG-3′ (antisense).

PCR products were resolved on a 2% agarose gel in the presence of RGB-4103 GelRed Nucleic Acid Stain.

Western blot analysis

RD and Rh30 rhabdomyosarcoma cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. Cells were seeded and subsequently treated with varying concentration of panobinostat or vorinostat for 3, 6, 9, 12, or 24 hours or with 100 nm of siSp1, siSp3, siSp4 for 72 hours. Cells were lysed with high salt lysis buffer (with protease inhibitor cocktail) and quantitated with Bradford reagent. Lysates were then analyzed by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane by wet electroblotting. Membranes were then incubated with primary and then followed by secondary antibody. Western blot analysis was determined as described and Immobilon western chemiluminescence substrates (Millipore, Billerica, MA) were used to develop images captured on a Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR).

Small interfering RNA interference assay

RD and Rh30 rhabdomyosarcoma cells were seeded (1.2 x 10^5 per well) in six well plates in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and left to attach

for 24 hours. Knockdown of NR4A1 was carried out using LIpofectamine 2000 reagent according to the manufactures protocol. Small inhibitory RNAs and GL2 (non-specific oligonucleotide) were prepared and purchased Sigma-Aldrich (St. Louis MO). The siRNA complexes used in the study are as follows: siGL2-5', CGU ACG CGG AAU ACU UCG A; siMyc, SASI_Hs01_00222676; siSp1,SASI_Hs02_00333289; siSp3, SASI_Hs01_00211941; siSp4, SASI_Hs01_00114420

Generation and measurement of ROS

Cellular ROS levels were ascertained using the cell permeable probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2'7' dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen (Grand Island, NY). CM-H_2DCFDA is non-fluorescent until cleavage of the acetyl groups by intracellular esterases and oxidation that transpires within the cell. Following treatment of the cells for 12 or 24 hours with panobinostat or vorinostat (+/- 5mM GSH 3 h prior to treatment) for 72 hours, cells plated on a 6-well culture plate were trypsinized, neutralized, then loaded with 10µM of probe for 20 min, washed once with serum free medium, and then ROS was measured by flow cytometry using Accuri's C6 Flow Cytometer (Accuri, Ann Arbor, MI).

Orthotopic implantation in vivo studies

Male SCID/beige mice were subcutaneously implanted with 5 *10^6 RD cells suspended in Matrigel (BD Biosciences). At 12 days post-implantation, when

tumors were palpable, mice were begun on a treatment regimen of 17.5 mg/kg Panobinostat or vehicle (DMSO), dosed intraperitoneally. Mice were treated daily for four days, no treatment for two days, and then every other day for 10 days. Animals were examined every other day for tumor burden (approximated by external caliper measurements, where [(width)2 × length] / 2), animal weight, and overall well-being. At study end, animals were humanely sacrificed and tumors were harvested for analysis.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by student's *t* test. The results are expressed as means with error bars representing 95% confidence intervals for 3 experiments for each group unless otherwise indicated, and a *P* value less than 0.05 was considered statistically significant. All Statistical tests were 2-sided.

Results

Figure 1.

Figure 73.

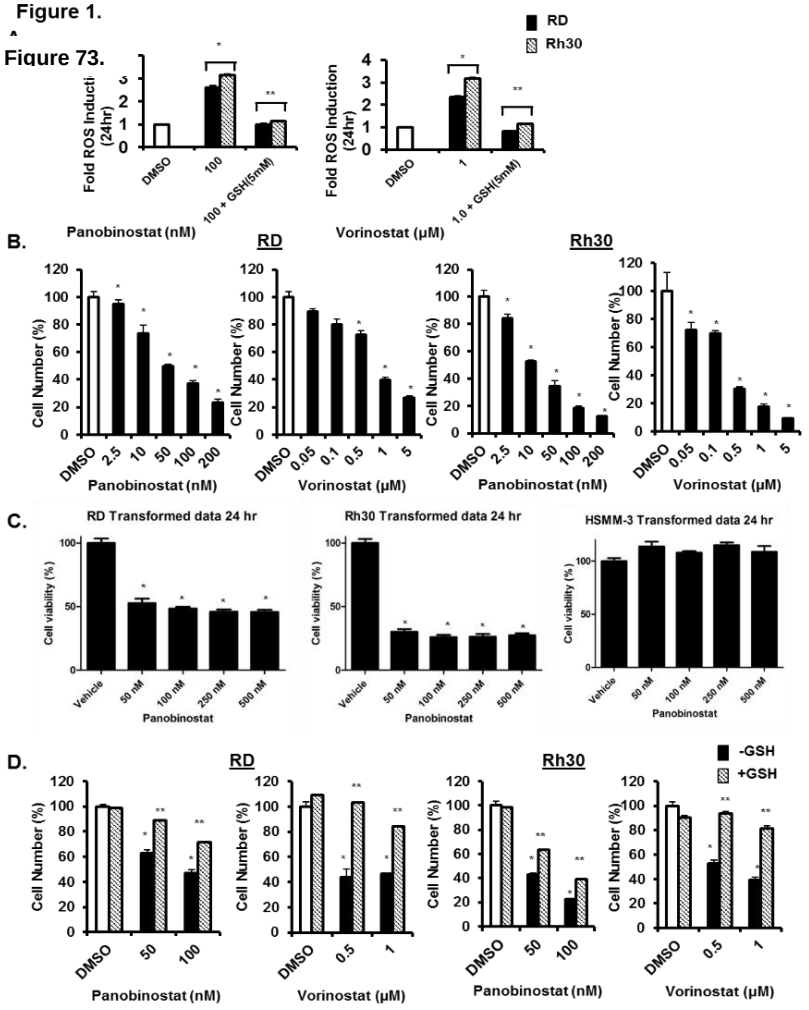

Figure 73. ROS-dependent inhibition of RMS cell growth by HDAC inhibitors. (A) RD and Rh30 cells were treated with DMSO, 100 nM panobinostat, or 1 μM vorinostat alone or in combination with 5 mM GSH, and ROS was determined fluorimetrically. (B) RD and Rh 30 cells were treated with panobinostat or varinostat and after 24 hr, cell growth was determined by counting cells in a Coulter counter. (C) Metabolic activity was also determined in RD, Rh30 and HSMM cells treated with panobinostat. (D) RD and Rh30 cells were treated with DMSO, panobinostat and vorinostat alone or in combination with 5 mM GSH, and cells were counted using a Coulter counter. Results are expressed means ± SE for at least 3 replicated determinations and significant ($p<0.05$) induction of ROS or growth inhibition (*) or reversal by GSH (**) is indicated.

The effectiveness of HDAC inhibitors such as panobinostat on the inhibition of patient-derived RMS xenografts was attributed, in part, to their activity as ROS

inducers. Figure 73A shows that both panobinostat and vorinostat induced ROS in RD and Rh30 cells and these responses were attenuated in cells cotreated with the HDAC inhibitors plus glutathione (GSH). Treatment of RD and Rh30 cells with different concentrations of both HDAC inhibitors also decreased proliferation of both cell lines with IC_{50} values of 96.7, 61.13 nM for panobinostat and 1.59, 1.1 μM for vorinostat in RD and Rh30 cell lines, respectively (Fig. 73B). Figure 73C shows that for the more potent HDAC inhibitor (panobinostat) we observed inhibition of metabolic activity using the MTT assay in RD and Rh30 cells, whereas in primary human skeletal muscle myoblasts (HSMMs) significant inhibition was not observed at concentrations as high as 500 nM, demonstrating specificity of the drug for the transformed cell lines. Finally, we also observed inhibition of RD and Rh30 cell growth by panobinostat and vorinostat was significantly attenuated after cotreatment with GSH (Fig. 73D) , indicating that induction of ROS by the HDAC inhibitors was important for their growth inhibitory effects.

Figure 74.

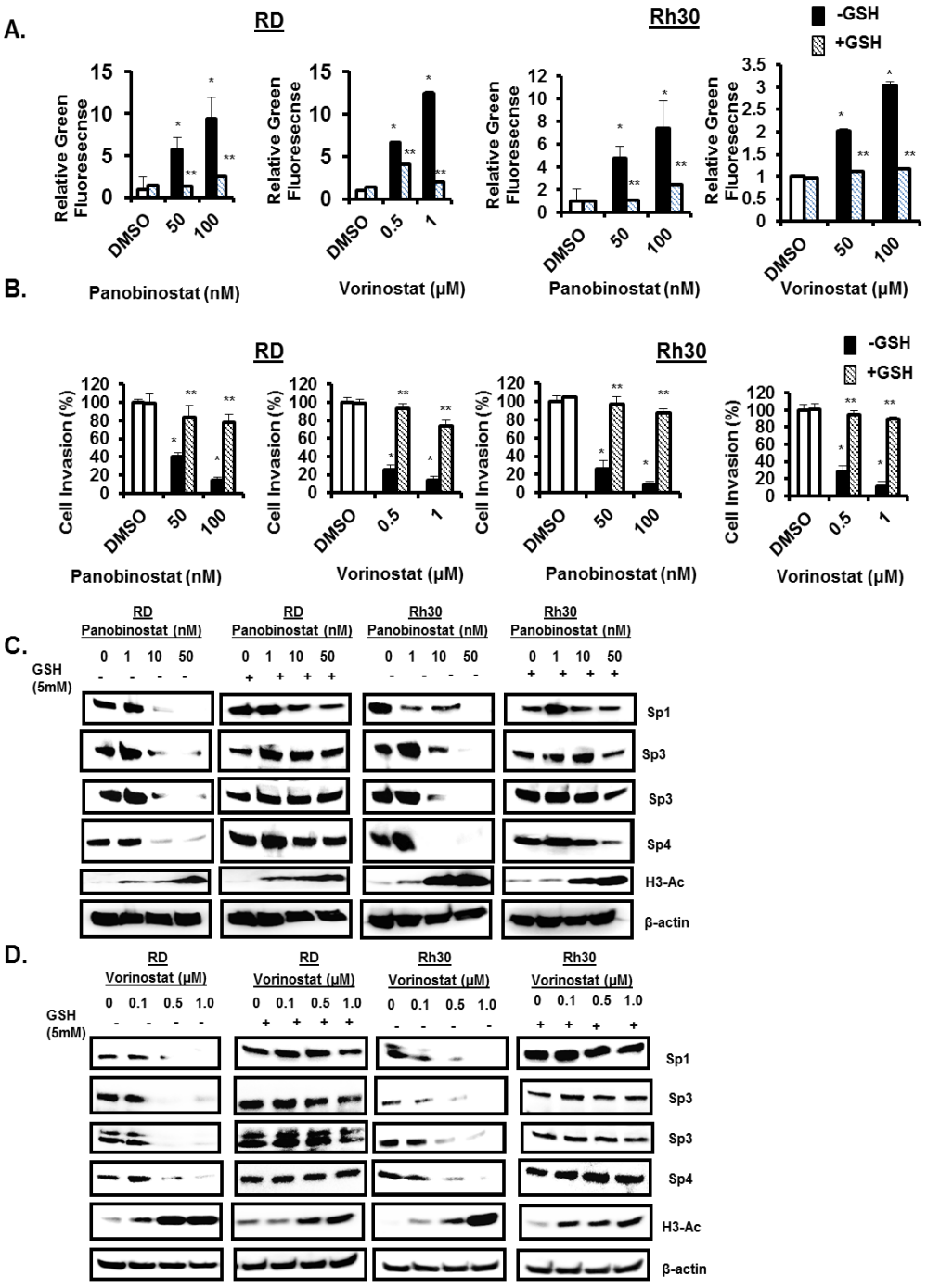

Figure 74. ROS-dependent induction of apoptosis, inhibition of invasion and downregulation of Sp proteins by panobinostat and vorinostat. RD and Rh30 cells were treated with DMSO, panobinostat or vorinostat alone or in combination with 5 mM GSH, and induction of Annexin V staining (A) or inhibition of invasion (B) were determined by fluorescence and a Boyden chamber assay, respectively. Results are expressed as means ± SE for at least 3 replicate determinations and significant ($p<0.05$) induction of Annexin V staining or inhibition of invasion (*) and inhibition by GSH (**) are indicated. RD and Rh30 cells were treated with panobinostat (C) or vorinostat (D) alone or in combination with 5 mM GSH for 24 hr, and whole cell lysates were analyzed by western blots.

Panobinostat and vorinostat also induced Annexin V staining, a marker of apoptosis in RD and Rh30 cells (Fig. 74A) and cotreatment with GSH attenuated this response. Panobinostat and vorinostat inhibited invasion in RD and Rh30 cells in a Boyden chamber assay (Fig. 74B) and this response was also attenuated in cells cotreated with GSH. Thus, ROS induction by both HDAC inhibitors resulted in the induction of apoptosis and inhibition of cell growth and invasion in RD and Rh30 cells. It has previously been reported that hydrogen peroxide and ROS-inducing anticancer agents decrease expression of Sp1, Sp3, and Sp4 transcription factors in pancreatic, colon and bladder cancer cells, and Sp1, Sp3, and Sp4 are also highly expressed in RMS and tumors (642,645,646,648,981-983). Panobinostat also decreased expression of Sp1, Sp3 (high and low molecular weight forms) and Sp4 in RD and Rh30 cells and cotreatment with GSH attenuated this response (Fig. 74C). We also observed similar effects in RD and Rh30 cells treated with vorinostat alone or in combination with GSH (Figure 74D) and both HDAC inhibitors increased histone-3 acetylation in RD and Rh30 cells which was unaffected by cotreatment with GSH. This suggests that the effects of panobinostat and vorinostat in this system are due to induction of ROS and are independent of their activity as inhibitors of histone deacetylation.

Figure 75.

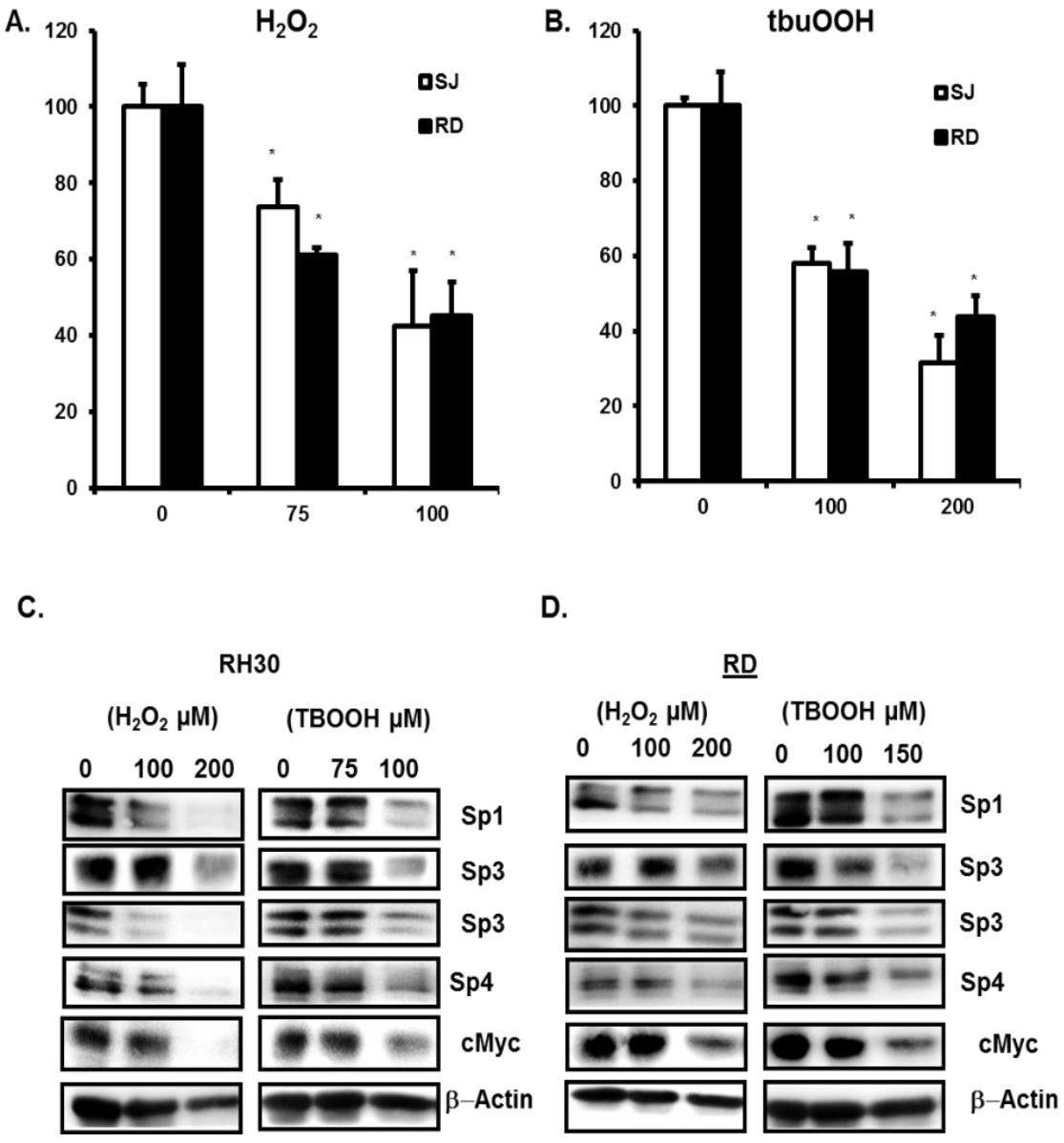

Figure 75. Hydrogen peroxide and *t*-butylhydroperoxide inhibit RMS cell growth and decreases Sp1, Sp3 and Sp4 in RMS cells. Rh30 and RD cells were treated with hydrogen perioxide (A) and *t*-butylhydroperoxide (B), and effects on cell proliferation were determined using a Coulter counter. Results are expressed as means ± SE for at least 3 replicate determinations and significant ($p<0.05$) growth inhibition (*) is indicated. Rh30 (C) and RD (D) cells were treated with hydrogen peroxide and *t*-butylhydroperoxide for 24 hr, and whole cell lysates were analyzed by western blots.

In pancreatic and bladder cancer cells, ROS such as hydrogen peroxide inhibits growth and decreased expression of Sp proteins (648,769). Figures 75A and 75B show that 75-150 μM hydrogen peroxide and 100-200 μM *t*-butylhydroperoxide, respectively, inhibited growth of RD and Rh30 cells. Moreover, the same concentrations of these compounds also decreased expression of Sp1, Sp3, and Sp4 proteins and cMyc (Fig. 75C and 75D) and these results were consistent with the effects we observed for panobinostat in RMS cells (Fig. 75C and 74D). Supplemental Figure 1 shows that after knockdown of Sp1, Sp3 and Sp4 by RNA interference there is inhibition of Rh30 and RD cell growth (Suppl. Fig. A-5A), induction of Annexin V staining (Suppl. Fig. A-5B) and decreased invasion in a Boyden chamber assay (Suppl. Fig. A-5C), demonstrating the important role of Sp TFs in the growth, survival and invasion of RMS cells and this was consistent with previous studies in RMS cells (976).

Figure 76.

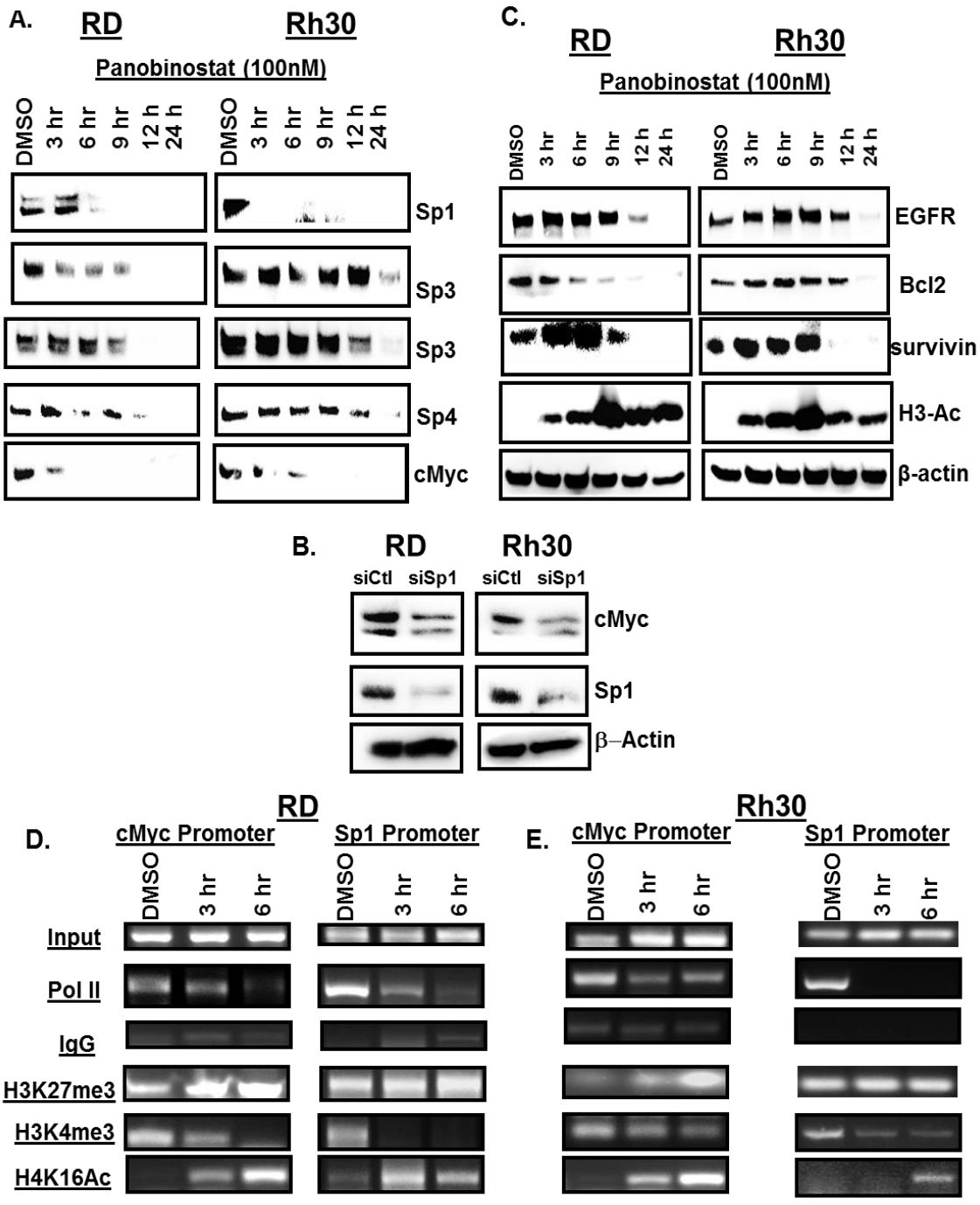

Figure 76. Panobinostat decreases Myc and Sp TFs through epigenetic pathways. RD and Rh30 cells were treated with panobinostat for different times, and whole cell lysates were analyzed for c-Myc and Sp TFs (A) and Sp-regulated genes (B) by western blot analysis. RD (C) and Rh30 (D) cells were treated with DMSO or panobinostat for 3 or 6 hr and analyzed in a ChiP assay using antibodies against pol II, IgG (control), and selected histone marks.

Hydrogen peroxide not only inhibits cancer cell growth but also induces genome wide shifts of repressor complexes from non-GC-rich to GC-rich promoters in SW480 cells resulting in rapid downregulation of Myc (781). Results illustrated in Figure 76A show that within 3 hr after treatment of RD and Rh30 cells with panobinostat, there was a significant decrease in expression of c-Myc and after 6 hr Myc protein levels were not detected for the 24 hr duration of the experiment. Interestingly, we also observed a rapid decrease in expression of Sp1 within 3-6 hr, whereas decreased expression of Sp3 and Sp4 proteins was observed only at longer time points. This suggests that like c-Myc, Sp1 expression may also be reduced by rapid ROS-dependent chromatin shifts that have previously been observed in colon and pancreatic cancer cells after treatment with hydrogen peroxide and PEITC (an ROS inducer), respectively (648,781). In contrast, the rate of degradation of Sp-regulated gene products including EGFR, bcl2, and survivin (Fig. 76B) was similar to that observed for Sp3 and Sp4 proteins and different from either c-Myc or Sp1. ROS-induced changes in histone marks in RD and Rh30 cells were investigated in a ChIP assay after treatment with panobinostat for 3 and 6 hr. In RD cells (Fig. 76C), there was a decrease in Pol II and the H3K4me3 activation mark on the c-Myc and Sp1 promoters and an increase in H4K16Ac. In Rh30 cells (Fig. 76D), there was also a decrease in Pol II and H3K4me3 and an increase in H4K16Ac on the c-Myc and Sp1 promoters as observed in RD cells; however, we also observed an increase in the H3K27me3 deactivation mark on the c-Myc promoter. These results are

consistent with previous studies on ROS-induced changes in histone marks (781) and with the exception of the increase in H4K16Ac (activation mark), the epigenetic changes correlate with the observed rapid decreases in c-Myc and Sp1 protein expression (Fig. 76A).

Figure 77.

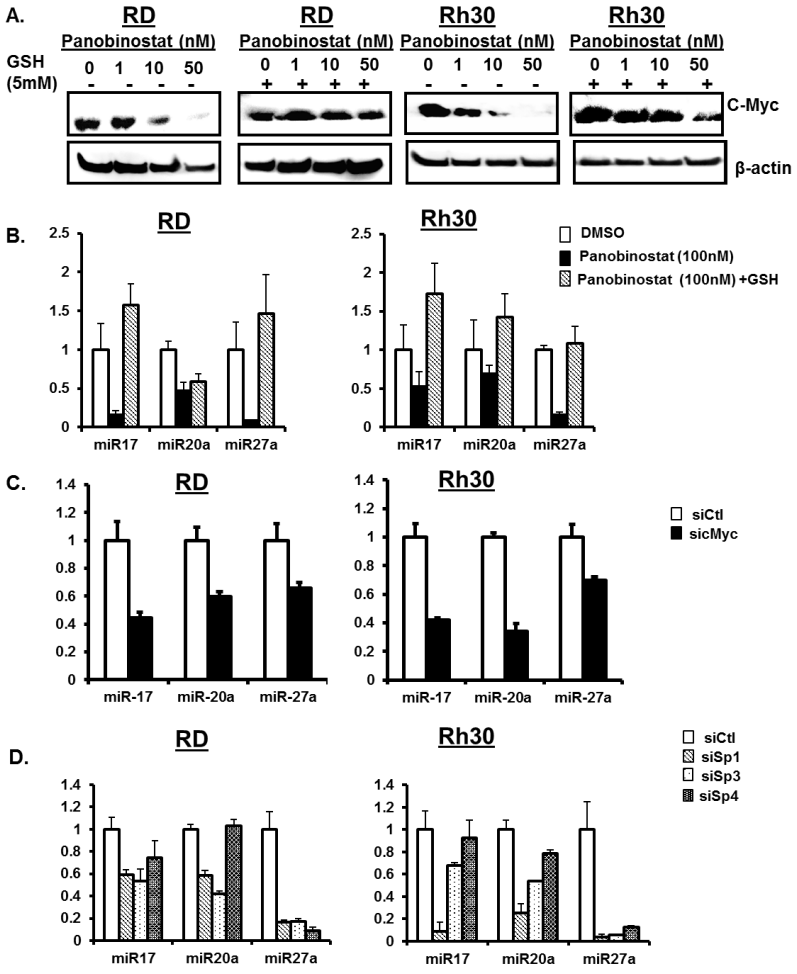

Figure 77. Panobinostat regulates c-Myc, and c-Myc and Sp TFs regulate miR-27a and miR-20/miR-17 in RMS cells. RMS cells were treated with panobinostat alone or in combination with GSH for 24 hr, and whole cell lysates were analyzed by western blots (A) or analyzed for miR expression (B) by real time PCR. RD and Rh30 cells were transfected sic-Myc (C) or siSp1, siSp3 and siSp4 (D), and miR expression was determined by real time PCR. Results (B-D) are expressed as means ± SE for 3 replicate determinations and significantly ($p<0.05$) decreased mir expression is indicated (*).

Previous studies in pancreatic cancers cells show that ROS-dependent downregulation of Myc also results in decreased expression of miR-27a and miR-20a/miR-17 and upregulation of the corresponding miR-regulated Sp repressors ZBTB34/ZBTB10 and ZBTB4, respectively (648). Results in Figure 77A show that after treatment of RD and Rh30 cells with panobinostat there was a decrease in c-Myc expression and this was attenuated in cells cotreated with panobinostat plus glutathione. Previous studies showed that miR-27a is expressed in RD and Rh30 cells (976), and treatment of these cell lines with 100 nM panobinostat decreased expression of miR-27a and this decrease was also attenuated in cells cotreated panobinostat plus glutathione (Fig. 77B). We also observed that miR-20a and miR-17 were expressed in both RMS cell lines and downregulation of these miRs with panobinostat was also inhibited in cells cotreated with the HDAC inhibitor plus glutathione. MiR-27a and miR20a/miR-17 are members of the miR-23a-27a-24-2 and miR-17-92 clusters, respectively, and there is evidence that both miR clusters are regulated by c-Myc (648,984-986), and transfection of RD and Rh30 cells with siMyc decreased miR-27a and miR-20a/miR17 expression (Fig. 77C). There is also some evidence that Sp1 regulates these same miR clusters, and knockdown of Sp1, Sp3 and Sp4 by RNAi decreased miR-27a and miR-20/miR-17 expression and the most dramatic effects were observed for miR-27a in both RD and Rh30 cells (Fig. 77B). These results could also be due in part to downregulationn of cMyc which can be regulated by Sp TFs.

Figure 78.

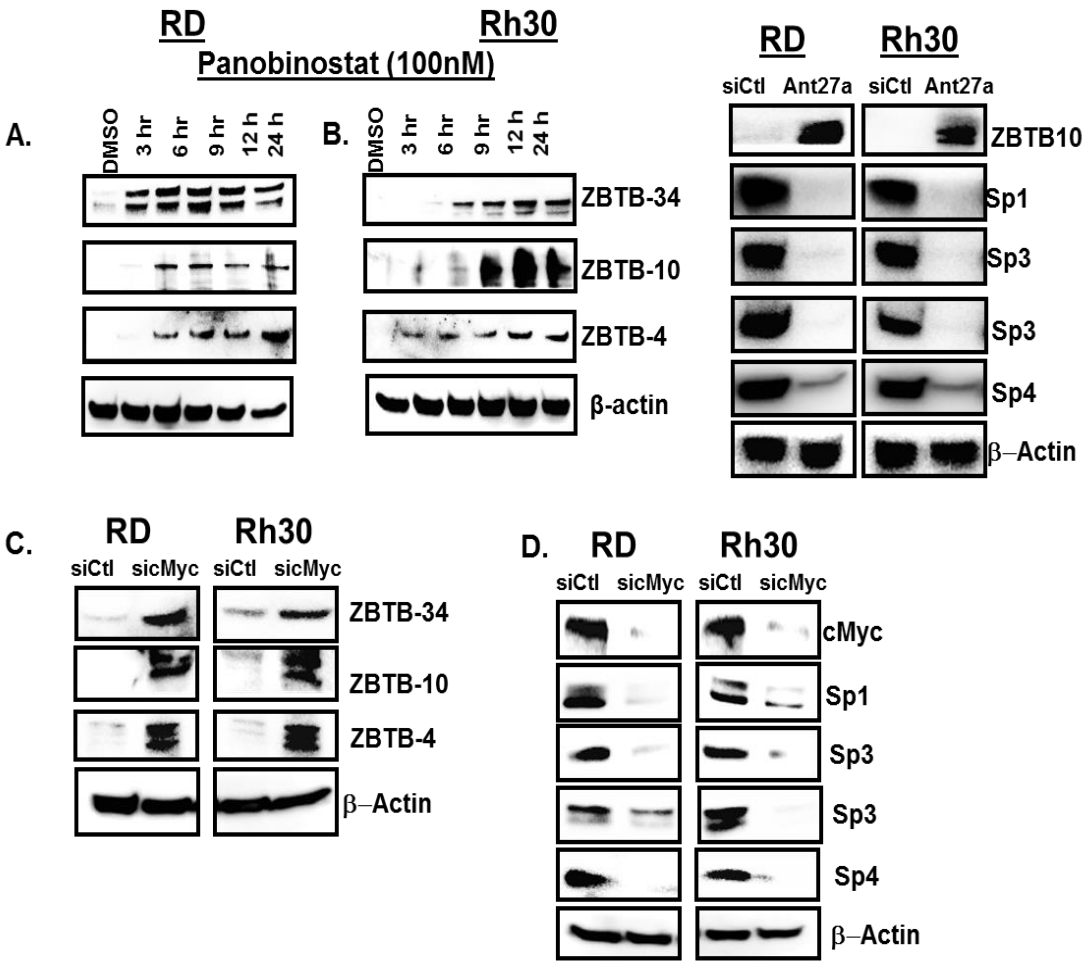

Figure 78. Panobinostat and c-Myc knockdown induce ZBTB transcriptional repressors; sic-Myc decreases Sp TFs in RMS cells. RD (A) and Rh30 (B) cells were treated with panobinostat for up to 24 hr, and whole cell lysates were analyzed by western vlots. RD and Rh30 cells were transfected with sic-Myc, and whole cell lysates were analyzed for ZBTB (D) and Sp (D) proteins by western blots.

Treatment of RD and Rh30 cells with panobinostat resulted in the induction of ZBTB10 and ZBTB34 (Figs. 78A and 78B) which are regulated by miR-27a, and ZBTB4 which is regulated by miR-20a/miR-17 (642,646,648,981-983). In addition, transfection of RD and Rh30 cells with siMyc increased expression of ZBTB10, ZBTB34, and ZBTB4 (Fig. 78C) and also decreased levels of Sp1, Sp3,

and Sp4 (Fig. 78D). This was also observed in the ERMS cell line SMS-CTR (Suppl. Fig. A-6)

Thus cMyc knockdown decreases expression of miRs, induces ZBTBs and decreases Sp TFs and thereby mimics the effects of ROS. *In vivo* studies with SCID mice bearing RD cells also showed that daily administration of panobinostat (17.5 mg/kg) decreased tumor volume (Fig. 79A) and growth (Fig. 79B), and analysis of the tumors showed that panobinostat also decreased Sp1, Sp3, Sp4 and c-Myc expression (Fig. 79C). These results are consistent with *in vitro* studies and demonstrate that the effectiveness of panobinostat as an anticancer agent in RMS cells is primarily due to targeting of Sp1, Sp3 and Sp4 transcription factors through an ROS-dependent mechanism involving downregulation of c-Myc and Myc-regulated miRs (Fig. 79D).

Figure 79.

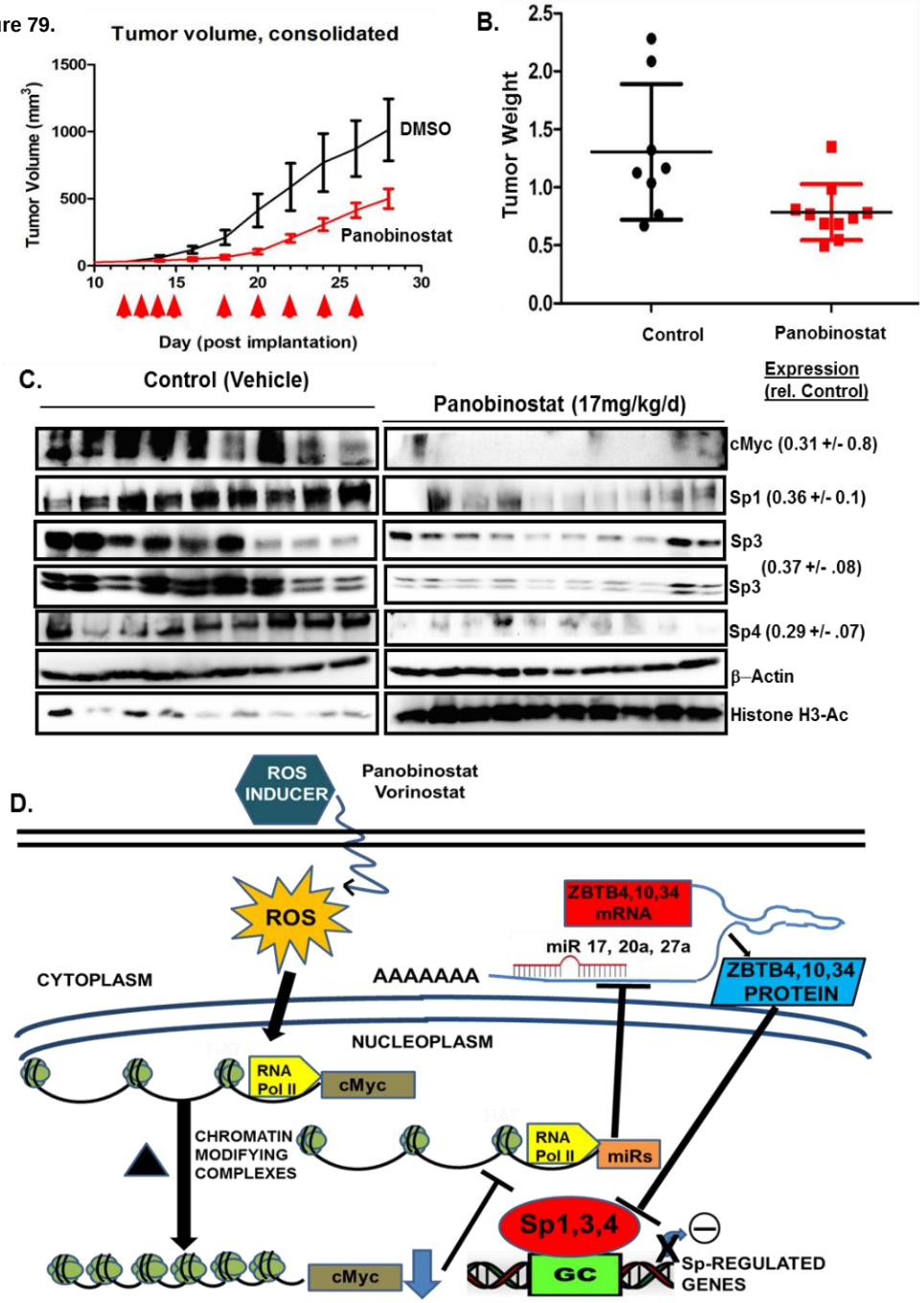

Figure 79. Panobinostat inhibits growth of RMS tumors. Panobinostat (17.5 mg/kg) was administered to SCID mice and tumor volumes (A) and weights (B) were determined. (C) Tumor lysates from control and panobinostat-treated mice were analyzed by western blots. (D) Mechanism of action of ROS-inducing HDAC inhibitors in RMS.

Discussion

Transformation of normal cells into cancer cells is a complex cell- and tissue-specific process that involves activation of oncogenes and inactivation of tumor suppressor genes, resulting in cancer cells that exhibit the typical hallmarks of cancer (60,987). In addition, there are many other genes that also significantly contribute to the cancer cell phenotype. These genes are typically overexpressed in tumor vs. non-tumor tissues and have been termed as non-oncogene addiction (NOA) genes and are excellent targets for mechanism-based antineoplastic agents. The transcription factor Sp1 plays a critical role in embryonic development but there is evidence that levels of Sp1 decrease with age in rodents and humans (988-990). In contrast, Sp1 is highly expressed in tumors from RMS patients and in many other cancers and high expression of Sp1 is a negative prognostic factor for patient survival, tumor recurrence or tumor grade (976,991-996).

Stable transduction of human skeletal muscle fibroblasts with PAX3-FOX01, telomerase and N-Myc resulted in formation of transformed cell lines similar to ARMS cells and both the genetically transformed and ARMS cells (997) expressed high levels of Sp1, Sp3 and Sp4 (976). Interestingly, transformation of the muscle fibroblasts dramatically increased expression of Sp1 and Sp3 but not Sp4 proteins which were highly expressed in the non-transformed cells (976); carcinogen/oncogene-induced transformation of human fibroblasts also

dramatically increased Sp1 expression but levels of Sp3 and Sp4 were not determined (998).

The pro-oncogenic functions of Sp1 have been reported in many different cell lines (reviewed in 996); however, it is also important to determine the role of Sp1, Sp3 and Sp4 since all three proteins are overexpressed in RMS and other cancer cell lines. Supplemental Figure 1 shows that after knockdown of Sp1, Sp3 and Sp4 in Rh30 and RD cells, there was a significant decreased in cell proliferation, induction of apoptosis (Annexin V staining), and inhibition of invasion indicating that all three Sp proteins are NOA genes in RMS cells. In previous studies, we demonstrated that tolfenamic acid-induced downregulation of Sp1, Sp3 and Sp4 inhibited growth, induced apoptosis and inhibited invasion in RMS cells, and this was accompanied by decreased expression of several pro-oncogenic Sp-regulated genes (976).

It was recently reported that mice bearing ERMS patient-derived xenografts were highly sensitive to drugs that induce oxidative stress and these drugs significantly inhibited tumors (650). We hypothesized that the efficacy of the ROS-inducing agents which included panobinostat was also due, in part, to repression of Sp1, Sp3 and Sp4. This hypothesis was based on previous studies showing that several ROS-inducing anticancer drugs including curcumin, phenethylisothiocyanate (PEITC), methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), GT-094 (a nitro-aspirin analog), celastrol and betulinic acid also

repressed Sp1, Sp3 and Sp4 expression (ROS-dependent) in pancreatic, bladder and colon cancer cells (642,645,646,648,981-983).

Therefore, we used the HDAC inhibitors panobinostat and vorinostat that are also ROS inducers (999) to investigate downregulation of Sp1, Sp3 and Sp4 in RMS cells in culture and in an *in vivo* model. Both panobinostat and vorinostat induced ROS, inhibited growth, induced apoptosis and inhibited invasion of RD and Rh30 cells, and all of these responses were attenuated after cotreatment with the antioxidant GSH (Figs. 73 and 74). Moreover, panobinostat also inhibited tumor growth in SCID mice bearing RD cells as a xenograft (Fig. 6C). In parallel studies, it was observed that panobinostat and vorinostat also decreased expression of Sp1, Sp3 and Sp4 in Rh30 and RD cells (Figs. 74C and 74D) and this response was attenuated after cotreatment with GSH. Moreover, treatment with hydrogen peroxide and *t*-butylhydroperoxide also decreased expression of Sp1, Sp3 and Sp4 as previously observed in other cell lines (769,982,1000). We also observed that panobinostat decreased c-Myc, Sp1, Sp3 and Sp4 proteins levels in tumors (Fig. 6C) and this paralleled the effects observed in cell culture (Fig. 74C). Thus, both knockdown of Sp TFs by RNA (Suppl. Fig. 1) and treatment with ROS inducers results in decreased expression of Sp1, Sp3 and Sp4, decreased growth and invasion, and induction of apoptosis *in vitro* and tumor growth *in vivo*. This suggests that the ROS-mediated repression of Sp TFs plays an important role in the antineoplastic effects of these HDAC inhibitors in RMS

cells and is consistent with the *in vitro* and *in vivo* efficacy of panobinostat in RMS patient-derived xenografts (650).

Previous studies show that ROS-dependent repression of Sp1, Sp3 and Sp4 was due to downregulation of miR-27a and/or miR-20a/miR-17-5 resulting in increased expression of the transcriptional repressors ZBTB10/ZBTB34 and ZBTB4, respectively (642,645,646,648,981-983). The ZBTB proteins are members of the POK family of transcriptional repressors (1001) and competitively bind and displace Sp TFs from GC-rich sites on Sp promoters and Sp-regulated gene promoters. Using panobinostat as a model ROS inducer, we show that this compound also decreases miR-27a and miR-20a/miR-17 in RD and Rh30 cells (ROS-dependent) (Fig. 77B) and this results in the induction of ZBTB10/ZBTB34 and ZBTB4 (Fig. 78A). These data also show that the high expression of Sp1, Sp3 and Sp4 in RMS cells is due, in part, to miR-dependent suppression of the ZBTB transcriptional repressors.

Treatment of colon cancer cells with hydrogen peroxide or pharmacological doses of ascorbate which induces hydrogen peroxide decreases expression of Sp1, Sp3 and Sp4 (1000). Under these same conditions, hydrogen peroxide induces extensive epigenetic and genetic changes in colon cancer cells due to genome-wide migration of chromatin-modifying complexes from non-GC-rich to GC-rich gene promoters (781). c-Myc was one of the genes rapidly downregulated by treatment with hydrogen peroxide, and similar results were observed for the ROS inducer PEITC in pancreatic cancer cells in which Sp1 was

also rapidly downregulated in some cell lines (648). Sp1, Sp3, Sp4 and c-Myc all have GC-rich promoters, and treatment of RD and Rh30 cells with panobinostat rapidly decreased expression of c-Myc and Sp1 proteins, whereas with the exception of the rapid decrease in expression of Sp3 (high MW band in RD cells), downregulation of Sp3 and Sp4 was primarily observed at later time points (Fig. 76A). Results of ChIP assays confirmed that treatment with panobinostat decreased pol II and the H3K4me3 activation mark in the GC-rich c-Myc and Sp1 promoters in RD and Rh30 cells, and the H3K27me3 inactivation mark was also increased on the c-Myc promoter only in Rh30 cells. Surprisingly, the H4K16Ac activation mark was increased by panobinostat in RD and Rh30 cells but decreased by PEITC-induced ROS in pancreatic cancer cells (648), suggesting that the epigenetic effects of ROS inducers are cell context-dependent and this is currently being investigated.

The importance of the ROS-dependent decrease in c-Myc is that there are E-box and GC-rich elements in the miR-23a~27A~24-2 and mir-17-92 gene cluster promoters (984-986) and knockdown of c-Myc or Sp TFs in RMS cells significantly decreased expression of miR-27a and miR-20a/miR-27 (Figs. 77C and 77D). It was also observed for the first time that Sp1, Sp3 and Sp4 play a role in expression of miR-27a and this paralleled the highly effective downregulation of miR-27a in RMS cells treated with HDAC inhibitors (Fig. 77B).

In summary, results of this study show that HDAC inhibitors that induce ROS are highly effective inhibitors of RMS cell in culture and tumor growth in

mice and the effects in vitro were independent of HDAC inhibition. This complements results obtained with HDAC inhibitors using tumor-derived xenografts and genomic analysis, thus confirming the potential efficacy of ROS inducers for RMS chemotherapy (650). This study demonstrates that panobinostat and vorinostat decrease expression of Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes by ROS-dependent epigenetic downregulation of c-Myc and Sp1 which in turn decrease expression of miR-27a and miR-20a/miR-17, resulting in the induction of the ZBTB transcriptional repressors (Fig. 79D). The study demonstrates why ROS inducers are highly effective for treating RMS since this pathway leads to a cascade of events leading to downregulation of Sp TFs and pro-oncogenic Sp-regulated genes. ROS inducers and other drugs targeting Sp TFs represent promising new approaches for RMS chemotherapy; moreover, since miRs that repress ZBTB repressors have been detected in serum (1002,1003), this may also provide a biomarker for monitoring treatment efficacy.

CHAPTER V

NR4A1 ANTAGONISTS INHIBIT β1-INTEGRIN-DEPENDENT BREAST CANCER CELL MIGRATION*

Introduction

Cell adhesion and attachment are essential for tissue integrity and cellular homeostasis, and the heterodimeric integrin cell surface receptors play a critical role in these processes (838-840). There are 18 different α subunits and 8 different β subunits that form 24 αβ-integrin receptor heterodimers, and the large 12-member β1-integrin subgroup bind multiple extracellular matrix (ECM) molecules to activate multiple intracellular pathways and also induce cross talk with other signaling systems (838-840). The functions of integrin heterodimers are highly tissue specific, and many human pathologies also involve integrin signaling (reviewed in 841,842). β1-Integrin is highly expressed in most tumors and is associated with a negative prognostic significance such as overall and disease-free survival, recurrence, and metastasis for head and neck and squamous cell carcinoma, melanoma, lung, breast, prostate, laryngeal, and pancreatic cancers (843-848,851-854,858,861).

*Reprinted with permission from "NR4A1 Antagonists Inhibit β1-Integrin-Dependent Breast Cancer Cell Migration." By Hedrick E, Lee SO, Doddapaneni 3, Singh M, Safe S. 2016 Molecular and Cellular Biology, Volume 36: pp1383-94. COPYRIGHT [2016} American Society for Microbiology

A recent immunostaining study of 225 breast invasive ductal carcinomas (IDCs) showed that β1-integrin was overexpressed in 32.8% of patients with IDCs. Numerous studies show that focal adhesion kinase (FAK) which is downstream from β1-integrin is also a negative prognostic factor for breast cancer patients (1004-1006). The important functional role of β1-integrin has been demonstrated in mouse models expressing *erbB2* under the control of the mouse mammary tumor virus and crossed with mammary tissue-specific β1-integrin-deficient mice. These mice exhibit a decrease in tumor volume, increased apoptosis, and decreased lung metastasis compared to animals expressing wild-type β1-integrin (862,1007,1008). Although small molecules, peptides, and antibodies that inhibit β1-integrin signaling have been developed, clinical agents that target β1-integrin for cancer chemotherapy are not currently available.

The orphan nuclear receptor 4A1 (NR4A1) (also called TR3 or Nur77) is overexpressed in breast cancer and other tumors, and functional studies show that NR4A1 exhibits prooncogenic activity (reviewed in 788). Studies in this laboratory have characterized a series of 1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl)methane (C-DIM) analogs that bind NR4A1 and act as receptor antagonists to inhibit growth and induce apoptosis in several cancer cell lines and in tumors from mouse xenografts (803-806,812,904). A recent study demonstrated functional interactions between NR4A1 and transforming growth factor β (TGF-β) and in estrogen receptor (ER)-negative MDA-MB-231 cells, knockdown of NR4A1 decreased migration and also inhibited TGF-β-induced

migration of this cell line (822). Results of gene array studies in pancreatic cancer cells identified β*1-integrin* as a potential NR4A1-regulated gene (805). In this study, we demonstrate that NR4A1 regulates β1-integrin expression and β1-integrin-dependent migration of breast cancer cells, and this is accompanied by decreased expression of β3-integrin. In MDA-MB-231 cells, results of our studies show that both constitutive and TGF-β-induced migration are dependent on nuclear and extranuclear NR4A1-regulated pathways, respectively. C-DIM/NR4A1 antagonists inhibit NR4A1-dependent expression of β1- and β3-integrins and other prooncogenic NR4A1-regulated genes and pathways and represent a novel class of mechanism-based anticancer agents**.**

Materials and Methods

Cell lines and antibodies

SKBR3, MDA-MB-231, and MCF-7 breast cancer cells were purchased from American Type Culture Collection (Manassas, VA). The cells were maintained at 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)–Ham's F-12 medium with 10% fetal bovine serum with antibiotic. NR4A1 antibody was purchased from Novus Biologicals (Littleton, CO). TGF-β was purchased from BD Biosystems (Bedford, MA). β-Actin antibody, Dulbecco's modified Eagle's medium, RPMI 1640 medium, and 36% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Hematoxylin

was purchased from Vector Laboratories (Burlingame, CA). β3-Integrin, phosphorylated focal adhesion kinase (p-FAK), FAK, axin 2, leptomycin B, and NR4A1 immunofluorescent antibody were purchased from Cell Signaling Technologies (Manassas, VA). β1-Integrin antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA), p84 antibody was purchased from GeneTex (Irvine, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Biotium (Hayward, CA).

Cell adhesion assay

SKBR3, MDA-MB-231, and MCF-7 cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium–Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. The cells were seeded and subsequently treated with various concentrations of 1,1-bis(3′-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH) or *p*-carboxymethylphenyl (1,1-bis(3′-indolyl)-1-(*p*-carboxymethylphenyl)methane [DIM-C-pPhCO_2Me]) for 24 h or 1 h prior to treatment with TGF-β (5 ng/ml) (4-h cotreatment) or without TGF-β or with 100 nM siβ1-integrin (small interfering RNA against β1-integrin) or siNR4A1 for 48 h. The cells were trypsinized, counted, and then placed for 90 min on BD BioCoat human fibronectin cellware 24-well plates (BD Biosciences, Bedford, MA). The medium was then aspirated, and the wells were gently washed with phosphate-buffered saline (PBS) and stained with 0.5% crystal violet stain. The cells were then

counted for adhesion to fibronectin. Wells coated with bovine serum albumin (BSA) and poly-l-lysine were used as negative controls.

Boyden chamber assay

SKBR3, MDA-MB-231, and MCF-7 cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium–Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. The cells were seeded and subsequently treated with various concentrations of DIM-C-pPhOH or DIM-C-pPhCO_2Me for 24 h or 1 h prior to treatment with TGF-β (5 ng/ml) (4 h cotreatment) or without TGF-β or with 100 nM siβ1-integrin, siNR4A1, siSp1 (Sp1 stands for specificity protein 1), or sip300 for 48 h. The cells were trypsinized, counted, placed in 24-well 8.0-μm-pore ThinCerts from BD Biosciences (Bedford, MA), allowed to migrate for 24 h, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted.

Real-time PCR

RNA was isolated using Zymo Research *Quick-RNA* MiniPrep kit (Irvine, CA). Quantification of mRNA (β1-integrin, β3-integrin) was performed using Bio-Rad iTaq universal SYBR green one-step kit (Richmond, CA) using the manufacturer's protocol with real-time PCR. TATA binding protein (TBP) mRNA was used as a control to determine relative mRNA expression.

I*mmunoprecipitation*

MDA-MB-231 cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium–Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and allowed to attach for 24 h. The medium was then changed to DMEM–Ham's F-12 medium containing 2.5% charcoal-stripped fetal bovine serum, and either dimethyl sulfoxide (DMSO) or TGF-β (5 ng/ml) was added for 4 h (after pretreatment with leptomycin B [20 nM] for 24 h or pretreatment with 20 μM DIM-C-pPhOH or DIM-C-pPhCO_2Me or no pretreatment). Protein A Dynabeads were prepared, and binding of antibody with protein and protein-protein interactions were isolated by Life Technologies immunoprecipitation kit using Dynabeads coated with protein A (Grand Island, NY) following the manufacturer's protocol. Protein-protein interactions of interest were determined by Western blot analysis.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. SKBR3 and MDA-MB-231 cells were treated with DMSO, DIM-C-pPhOH, or DIM-C-pPhCO_2Me (15 or 20 μM) for 24 h. The cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by the addition of 0.125 M glycine. After the cells were washed twice with phosphate-buffered saline, the cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (~200 to 1,500 bp).

The sonicated chromatin was immunoprecipitated with normal IgG, p300 (Santa Cruz), siSp1 (Abcam), NR4A1 (Novus Biologicals), or RNA polymerase II (Pol II) (Active Motif) antibodies and protein A-conjugated magnetic beads at 4°C overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. DNA was prepared by proteinase K digestion followed by PCR amplification. The primers for detection of the β1-integrin promoter region were 5′-TCACCACCCTTCGTGACAC-3′ (sense) and 5′-GAGATCCTGCATCTCGGAAG-3′ (antisense), and the primers for detection of the β3-integrin promoter region were 5′-TCTCAGGCGCAGGGTCTAGAGAA-3′ (sense) and 5′-TCGCGGCGCCCACCGCCTGCTCTACGCT-3′ (antisense). PCR products were resolved on a 2% agarose gel in the presence of RGB-4103 GelRed nucleic acid stain.

Nuclear/cytosolic extraction

MDA-MB-231 cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium–Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. The medium was then changed to DMEM–Ham's F-12 medium containing 2.5% charcoal-stripped fetal bovine serum, and either DMSO or TGF-β (5 ng/ml) was added for 4 h (after pretreatment with 20 nM leptomycin B for 24 h or pretreatment with 20 μM DIM-C-pPhOH or DIM-C-pPhCO_2Me or no pretreatment). Nuclear and cytosolic fractions were then isolated using Thermo Scientific NE-PER nuclear and cytoplasmic extraction kit (Rockford, IL) according to the manufacturer's protocol.

Fractions were then analyzed by Western blotting. GAPDH and p84 were used as cytoplasmic and nuclear positive controls, respectively.

Immunofluorescence

MDA-MB-231 (1.0×10^5 per well) were seeded in two-well Nunc Lab-Tek chambered borosilicate cover glass slides (no. 1 borosilicate cover glass) from Thermo Scientific and were allowed to attach for 24 h. The medium was then changed to DMEM–Ham's F-12 medium containing 2.5% charcoal-stripped fetal bovine serum, and either DMSO or TGF-β (5 ng/ml) was added for 4 h (after pretreatment with leptomycin B [20 nM] for 24 h or pretreatment with 20 μM DIM-C-pPhOH or DIM-C-pPhCO_2Me or no pretreatment). The cells were then treated with fluorescent NR4A1 primary antibody [Nur77 (D63C5) XP], and immunofluorescence was observed according to Cell Signaling Technology's immunofluorescence protocol. 4′,6′-Diamidino-2-phenylindole (DAPI) staining was observed using Hoechst staining according to Biotium's apoptotic and necrotic assay kit by following the manufacturer's protocol. The cells were visualized by microscopy (Advanced Microscopy), and NR4A1 localization was determined by green fluorescence. DAPI was used to stain the nucleus, and images were taken sequentially of NR4A1, DAPI, and then merged (806,812,904).

Western blot analysis

SKBR3, MDA-MB-231, and MCF-7 cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium–Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. The cells were transfected with 100 nM siβ1-integrin, siNR4A1, siSp1, or sip300 for 72 h or treated with various C-DIM compounds. Cell lysates were analyzed by Western blotting as described previously (806,812,904).

Small interfering RNA interference assay

Small interfering RNA (siRNA) experiments were conducted as described previously. The siRNA complexes used in the study are as follows: siGL2-5′, CGU ACG CGG AAU ACU UCG A; siNR4A1, SASI_Hs02_00333289[1], SASI_Hs02_00333290[2]; siβ1-integrin, SASI_Hs02_00333437[1], SASI_Hs01_00159474; siSp1, SASI_Hs02_003; sip300, SASI_Hs01_00052818.

Triple-negative breast cancer (TNBC) orthotopic xenograft studies

Female BALB/c nude mice (6 to 8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA) and maintained and treated as previously described (904). Tumor volumes and tumor weights were determined as previously described (904). Tumor lysates were obtained and analyzed by Western blotting.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by Student's *t* test. The results are expressed as means with error bars representing 95% confidence intervals (95% CIs) for at least three experiments for each group unless otherwise indicated. A *P* value of <0.05 was considered statistically significant. All statistical tests were two sided.

Results

Figure 80.

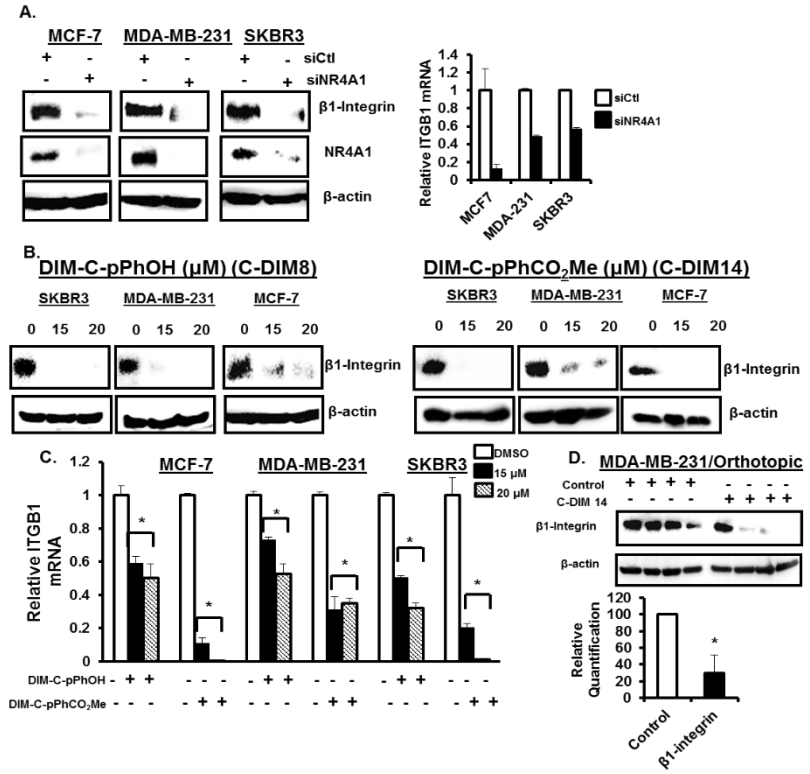

Figure 80. NR4A1 regulates β1-integrin expression in breast cancer cells and tumors. (A) Breastcancer cells were transfected with siNR4A1 and cell extracts were analyzed for protein and mRNA expression by western blots or real time PCR, respectively, as outlined in the Materials and Methods. Breast cancer cells were treated with DMSO, DIM-C-pPhOH or DIM-C-pPhCO2Me for 24 hr and extracts were analyzed for protein (B) or mRNA (C) levels by western blots and real time PCR, respectively, as outlined in the Materials and Methods. (D) Cell lysates from tumors (MDA-MB-231 orthotopic) (30) derived from animals treated with corn oil (control) or DIM-C-pPhCO2Me (C-DIM-14; 40 mg/kg/d) were analyzed by western blots and decreased protein expression was determined and normalized the β-actin protein loading control. Quantified data are presented as means ± SE (at least 3 replicates) and significant (*P*<0.05) decreases are indicated (*).

Figure 81.

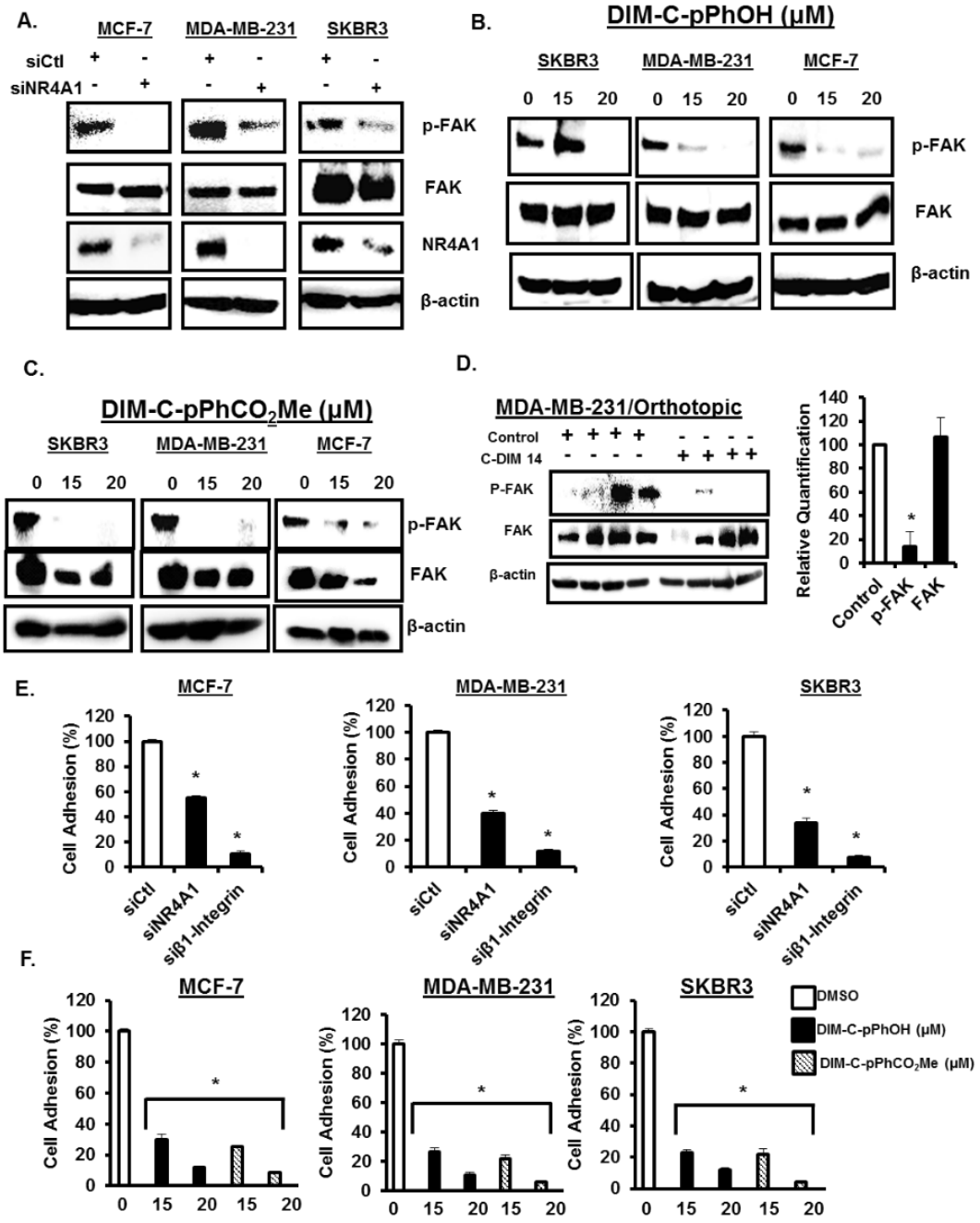

Figure 81. NR4A1 regulates β1-integrin-dependent responses. Breast cancer cells were transfected with siNR4A1 (A), treated with DMSO and DIM-C-pPhOH (B) or DIM-C-pPhCO2Me (C) for 24 hr, and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (D) Tumor lysates from mice (MDA-MB-231 orthotopic derived (30) treated with corn oil or DIM-C-pPhCO2Me (40 mg/kg/d) were analyzed by western blots and quantitated as outlined in Figure 1D. The effects of siNR4A1 and siβ1- integrin (E) or DIM-C-pPhOH and DIM-C-pPhCO2Me (F) on fibronectin-induced adhesion of breast cancer cells was determined as outlined in the Materials and Methods. Results (D-F) are means ± SE (at least 3 replicates) and a significant (*P*<0.05) decrease indicated (*). Western blots in Figures 1 and 2 were derived from the same experiment showing effects on β1-integrin (Fig. 80) and β1-integrin-regulated responses (Fig. 81).

NR4A1 regulates β1-integrin expression

β1-Integrin is expressed in ER-positive MCF-7, ER-negative MDA-MB-231, and *erbB2*-overexpressing SKBR3 breast cancer cells, and knockdown of NR4A1 (siNR4A1) by RNA interference (RNAi) decreased expression of β1-integrin protein and mRNA (Fig. 80A). Previous studies identified 1,1-bis(3′-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH; C-DIM8) and 1,1-bis(3′-indolyl)-1-(*p*-carboxymethylphenyl)methane (DIM-C-pPhCO_2Me; C-DIM14) as NR4A1 ligands that act as antagonists in breast and other cancer cell lines (804-806,812,904), and both compounds also decreased expression of β1-integrin protein (Fig. 80B) and mRNA (Fig. 80C) in MCF-7, MDA-MB-231, and SKBR3 cells. Moreover, Western blot analysis of tumor lysates from mice bearing MDA-MB-231 cells (orthotopic) (904) showed that DIM-C-pPhCO_2Me significantly decreases β1-integrin protein expression (Fig. 80D). β1-Integrin regulates phosphorylation of FAK (p-FAK), and transfection of MCF-7, MDA-MB-231, and SKBR3 cells with siNR4A1 (Fig. 81A) or treatment with DIM-CpPhOH (Fig. 81B) or DIM-C-pPhCO_2Me (Fig. 81C) decreased phosphorylation of FAK. In addition, results from the *in vivo* orthotopic study (904) showed that p-FAK is decreased in tumors from mice bearing MDA-MB-231 cells and treated with DIM-C-pPhCO_2Me (Fig. 80D). Fibronectin-induced cell adhesion is also a prototypical β1-integrin-regulated response, and cell adhesion was significantly decreased in MCF-7, MDA-MB-231, and SKBR3 cells after transfection with siNR4A1 (Fig. 81E) or after treatment with DIM-C-pPhOH or DIM-C-pPhCO_2Me (Fig. 81F). For a positive control, we showed

that knockdown of β1-integrin (siβ1-integrin) by RNA also decreased cell adhesion (Fig. 80E) (Suppl. Fig. A-7).

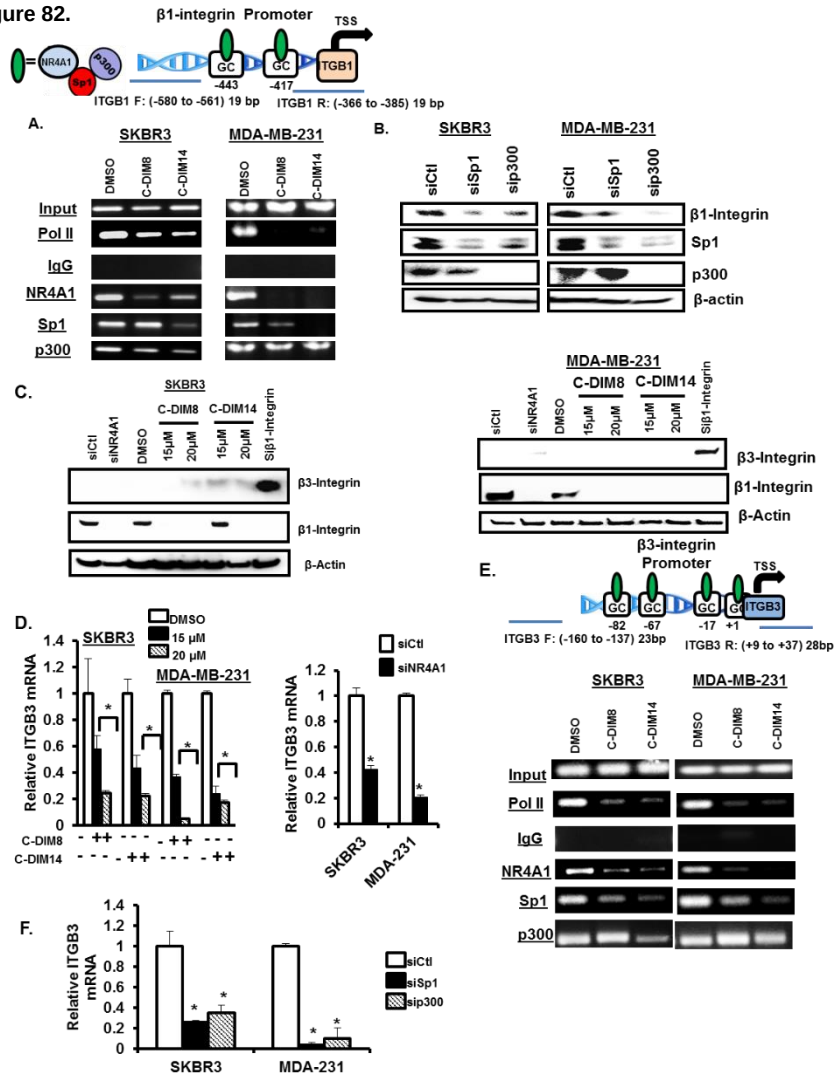

Figure 82. Role of NR4A1/p300/Sp1 in regulation of β1- and β3-integrin. (A) Analysis of polII, NR4A1, Sp1 and p300 binding to the β1-integrin promoter was determined in a ChIP assay using primers as indicated. (B) Cells were treated with oligonucleotides that knockdown Sp1 (siSp1) and p300 (sip300), and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (C) Cells were transfected with siNR4A1 or treated with DIM-C-pPhOH (C-DIM8) or DIM-C-pPhCO2Me (C-DIM14), and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (D) Cells were transfected with siNR4A1 or treated with C-DIM8 or C-DIM14 and effects on β3-integrin (ITGB3) mRNA levels were determined. The treatments significantly (*P*<0.05) decrease mRNA levels. (E) Analysis of polII, NR4A1, Sp1 and p300 binding to the proximal GC-rich region of the β3-integrin promoter was determined in a ChIP assay as outlined in the Materials and Methods. (F) Cells were transfected with siSp1 and sip300 and analyzed by real time PCR for β3-integrin mRNA levels. Both oligonucleotides significantly (*P*<0.05) decreased β3-integrin mRNA levels.

Mechanisms of NR4A1 regulation of β1-integrin and β3-integrin

NR4A1 regulates gene expression through direct interactions with genomic nerve growth factor Bα (NGFBα) response elements (NBRE) and Nur response elements (NuRE) or by interactions with specificity protein 1 (Sp1) bound to GC-rich promoter elements (1009,1010). NBRE and NuRE were not identified in the β1-integrin promoter, whereas two GC-rich sequences were located at −760 and −676 in the proximal region of the β1-integrin promoter (Fig. 82A). Previous studies show that NR4A1, Sp1, and the nuclear coregulatory gene p300 interact with the GC-rich region of the survivin promoter to regulate survivin gene expression (804). Using the more aggressive SKBR3 and MDA-MB-231 cells as models, cells were treated with dimethyl sulfoxide (DMSO), DIM-C-pPhOH, or DIM-C-pPhCO_2Me and analyzed in a chromatin immunoprecipitation (ChIP) assay using primers targeted to the GC-rich region of the β1-integrin promoter.

The results show that Pol II, NR4A1, Sp1, and p300 interact with the GC-rich promoter regions, and after treatment with DIM-C-pPhOH or DIM-C-pPhCO_2Me for 24 h, the band for Pol II was decreased in both cell lines (Fig. 82A), and this was consistent with decreased β1-integrin expression. Ligand-induced inactivation of NR4A1 also decreased NR4A1 binding to the promoter; however, changes in the Sp1 and p300 bands were somewhat variable and dependent on cell context and ligand. For example, the loss of p300 was observed in SKBR3 cells but not MDA-MB-231 cells, and it is possible that p300 was interacting with the *trans*-acting factors in the proximal region of the β1-integrin promoter. We

further investigated the roles of Sp1 and p300 in regulating β1-integrin expression in SKBR3 and MDA-MB-231 cells by RNAi, and knockdown of Sp1 (siSp1) and p300 (sip300) also decreased β1-integrin expression (Fig. 82B), suggesting that like survivin (804), NR4A1 regulates β1-integrin expression through a NR4A1/p300/Sp1 complex. p300 knockdown also decreases Sp1 expression, suggesting that p300 plays a role in regulating expression of this gene. These results do not exclude a role for other factors in NR4A1 regulation of β1-integrin, and this is currently being investigated. levels.

Previous reports show that inhibition of β1-integrin by RNAi or other β1-integrin inhibitors increases expression of β3-integrin resulting in enhanced metastasis (382,1011,1012). The β3-integrin promoter is also GC rich (1013) and therefore, we investigated the possible regulation of β3-integrin by NR4A1. Western blot analysis showed that constitutive β3-integrin protein levels were barely detectable and remained low after treatment with C-DIM/NR4A1 antagonists or siNR4A1 (Fig. 82C), whereas knockdown of β1-integrin by RNAi increased β3-integrin protein as previously reported (1012). There was more robust expression of β3-integrin mRNA in MDA-MB-231 and SKBR3 cells, and transfection of siNR4A1 or treatment with C-DIM/NR4A1 antagonists significantly decreased β3-integrin mRNA levels (Fig. 82D).

ChIP assays showed that NR4A1, Sp1, and p300 bound the proximal GC-rich region of the β3-integrin gene, and treatment with DIM-C-pPhOH or DIM-C-pPhCO_2Me decreased binding of Pol II, NR4A1, and Sp1 but differentially affected

p300 binding to the promoter. In addition, we also observed that knockdown of Sp1 (siSp1) or p300 (sip300) in MDA-MB-231 and SKBR3 cells decreased β3-integrin mRNA levels (Fig. 82E). These results demonstrate that NR4A1 regulates both β1- and β3-integrin expression, and in contrast to β1-integrin-specific inhibitors, NR4A1 antagonists downregulate expression of both β1- and β3-integrin. We also sucessfully demonstrate that the C-DIM/NR4A1 antagonists that act as nuclear inhibitors and keep NR4A1 nuclear in contrast to other known inhibitors of NR4A1 which induce mitochondrial translocation. The C-DIM/NR4A1 anatonists C-DIM8 and C-DIM14 (data not shown) both inhibit cell invasion in the absence or presence of leptomycin B (Fig. 83F). Thus this corroborates, along with the rest of Figure 83, the concept of the C-DIM/NR4A1 antagonists inhibiting β1-integrin-dependent migration/invasion by virtue of inhibitng NR4A1 from serving as a transcriptional activator of β1-integrin.

Figure 83.

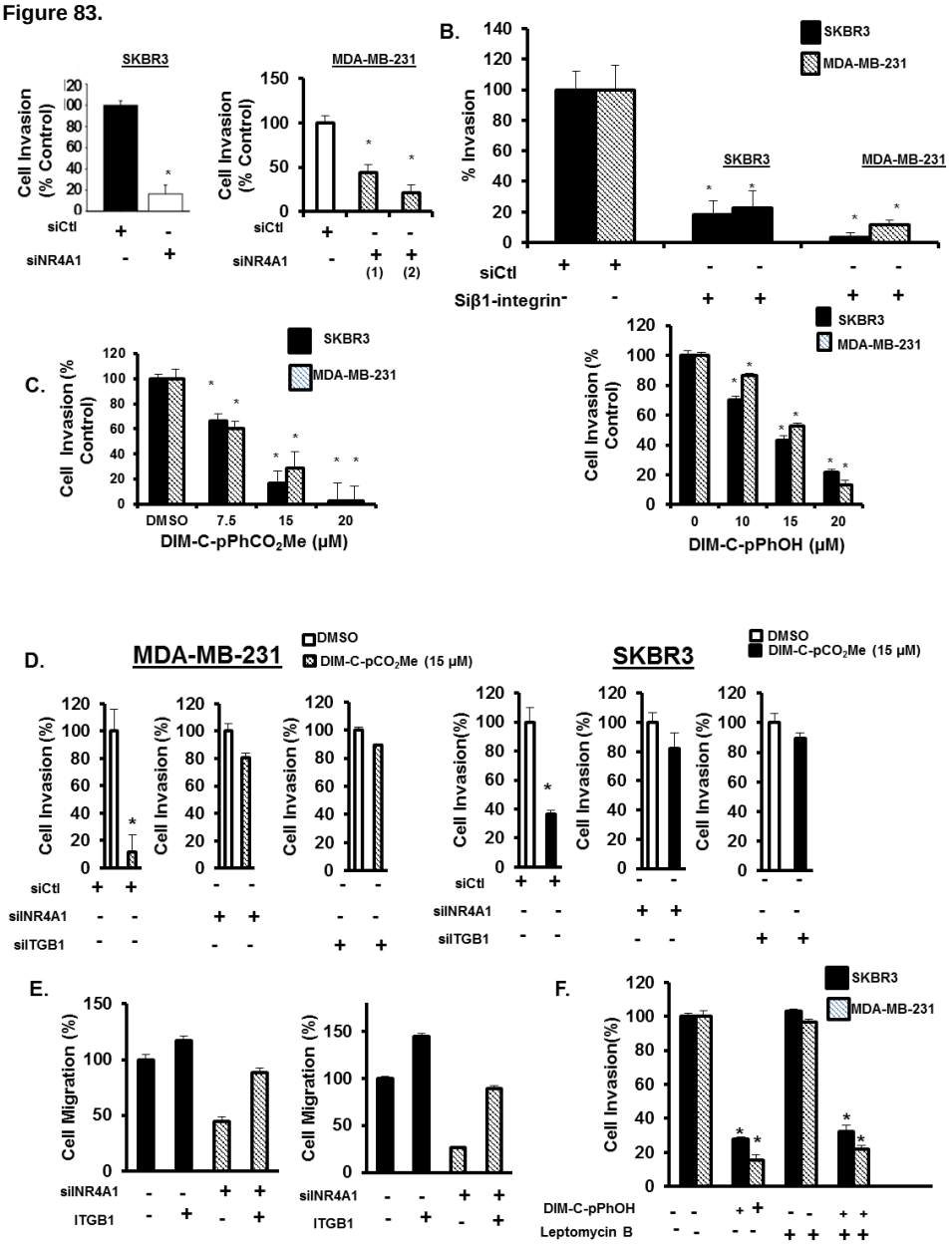

Figure. 83. NR4A1-regulates β1-integrin-dependent breast cancer cell migration. Cells were transfected with siNR4A1 (A), siβ1-integrin (B), or treated with DIM-C-pPhOH and DIM-C-pPhCO2Me (C), and DIM-C-pPhOH ± LMB (D), and breast cancer cell migration was determined in a Boyden chamber assay as outlined in the Materials and Methods. (E) Cells were transfected with a non-specific oligonucleotide (siCtl), siNR4A1, siβ1-integrin and treated with DIM-C-pPhCO2Me, and cell migration was determined in a Boyden chamber assay as outlined in the Materials and Methods. Cells were transfected with siNR4A1 alone (F) or treated with DIM-C-pPhOH/DIM-CpPhCO2Me in combination with β1-integrin (ITGB1) expression plasmid, and effects on cell migration were determined in a Boyden chamber assay as outlined in the Materials and Methods. Results are expressed as means ± SE for at least 3 replicates for each treatment group and significantly (*P*<0.05) decreased migration (*) or rescue by β1-integrin overexpression (**) are indicated.

Migration of MDA-MB-231 and SKBR3 cells: roles of NR4A1 and β1-integrin

Both MDA-MB-231 and SKBR3 cells undergo migration (constitutive) in a Boyden chamber assay in the absence of a stimulus. Transfection of these cells with siNR4A1 (Fig. 83A) or siβ1-integrin (Fig. 83B) decreased migration of both cell lines, and similar results were observed with two oligonucleotides targeting NR4A1 and β1-integrin. Treatment of SKBR3 and MDA-MB-231 cells with DIM-C-pPhOH (CDIM8) or DIM-C-pPhCO$_2$Me (CDIM14) also decreased migration (Fig. 83C), and the effects of DIM-C-pPhOH as an inhibitor of cell migration was not affected by cotreatment with leptomycin B (LMB), confirming that the inhibitory effects of this NR4A1 antagonist did not require nuclear export. We also investigated the role of NR4A1 in mediating DIM-C-pPhCO$_2$Me-dependent inhibition of migration of MDA-MB-231 and SKBR3 cells by knocking down NR4A1 and then treating with the NR4A1 antagonist DIM-C-pPhcO$_2$Me (Fig. 83E). Treatment of the NR4A1-depleted cells with DIM-C-pPhCO$_2$Me resulted in minimal inhibition of cell migration. Similar results were observed after treatment of β1-integrin-depleted cells with DIM-C-pPhCO$_2$Me, and we also observed that DIM-C-pPhOH did not inhibit invasion in cells depleted of NR4A1 or β1-integrin (Suppl. Fig. A-8). This would suggest that induction of β3-integrin after knockdown of β1-integrin (Fig. 82B) does not play a very significant role in cell migration using the Boyden chamber assay. Thus, inhibition of breast cancer cell migration by C-DIMs/NR4A1 antagonists is dependent on both NR4A1 and β1-integrin and consistent with regulation of β1-integrin by NR4A1. Overexpression of β1-integrin

in SKBR3 and MDA-MB-231 cells slightly increases cell migration, and in NR4A1-depleted cells which exhibit decreased migration, overexpression of β1-integrin significantly reverses this response (Fig. 83F). In addition, NR4A1 ligand-mediated inhibition of breast cancer cell migration was also rescued by β1-integrin overexpression (Fig. 83G), further confirming that β1-integrin-mediated migration is NR4A1 dependent. Previous studies show that C-DIMs specifically target nuclear NR4A1 and antagonism of NR4A1-dependent genes/responses are not affected by leptomycin B (LMB)-mediated inhibition of nuclear export (804), and results in Figure 4F show that LMB also did not affect DIM-C-pPhOH-depepndent inhibition of breast cancer cell migration. Thus, the constitutive or basal migration of SKBR3 and MDA-MB-231 cells in the absence of endogenous stimuli is linked to nuclear NR4A1 regulation of β1-integrin.

TGF-β-induced migration of MDA-MB-231 cells: role of extranuclear NR4A1

A recent study reported that TGF-β-induced migration of MDA-MB-231 cells was also NR4A1 dependent and involved a pathway associated with SMAD7 degradation resulting in activation of TGF-βR receptor 1 (TGF-βR1) (822). Treatment of MDA-MB-231 cells with 5 ng/ml TGF-β significantly induced cell migration (Fig. 84A) as previously described (822), and knockdown of NR4A1 or treatment with DIM-C-pPhOH or DIM-C-pPhCO2Me blocked TGF-β-induced migration and significantly decreased overall migration, similar to that observed after knockdown of NR4A1 or treatment with the NR4A1 antagonists alone (Fig. 83A to C). TGF-β-induced migration was inhibited after cotreatment with the TGF-

βR1 inhibitor ALK5i and also the nuclear export inhibitor LMB, and ALK5i had no effect on endogenous cell migration (data not shown). Analysis of cytosolic and nuclear extracts show that TGF-β induced expression and nuclear export of NR4A1 which was blocked by LMB (Fig. 84B) indicating that TGF-β-induced migration requires cytosolic NR4A1, whereas constitutive migration which is not inhibited by ALK5i is due to nuclear NR4A1-dependent regulation of β1-integrin. We also examined SMAD7 expression and observed minimal endogenous expression in MDA-MB-231 and SKBR3 cells, and TGF-β increased SMAD7 only in SKBR3 cells (Fig. 84C). In contrast, cotreatment with TGF-β plus LMB, CDIM8, or CDIM14 dramatically increased SMAD7 protein expression of both cell lines, suggesting that nuclear localization of NR4A1 inhibits degradation of SMAD7, which is a cytosolic protein. These results are consistent with previous studies, suggesting that NR4A1 (cytosolic) plays a role in proteasome-dependent degradation of SMAD7. Immunostaining of NR4A1 in MDA-MB-231 cells confirms that NR4A1 is nuclear, and treatment with TGF-β induces nuclear export of this receptor, and this is blocked by LMB (Fig. 84C).

Figure 84.

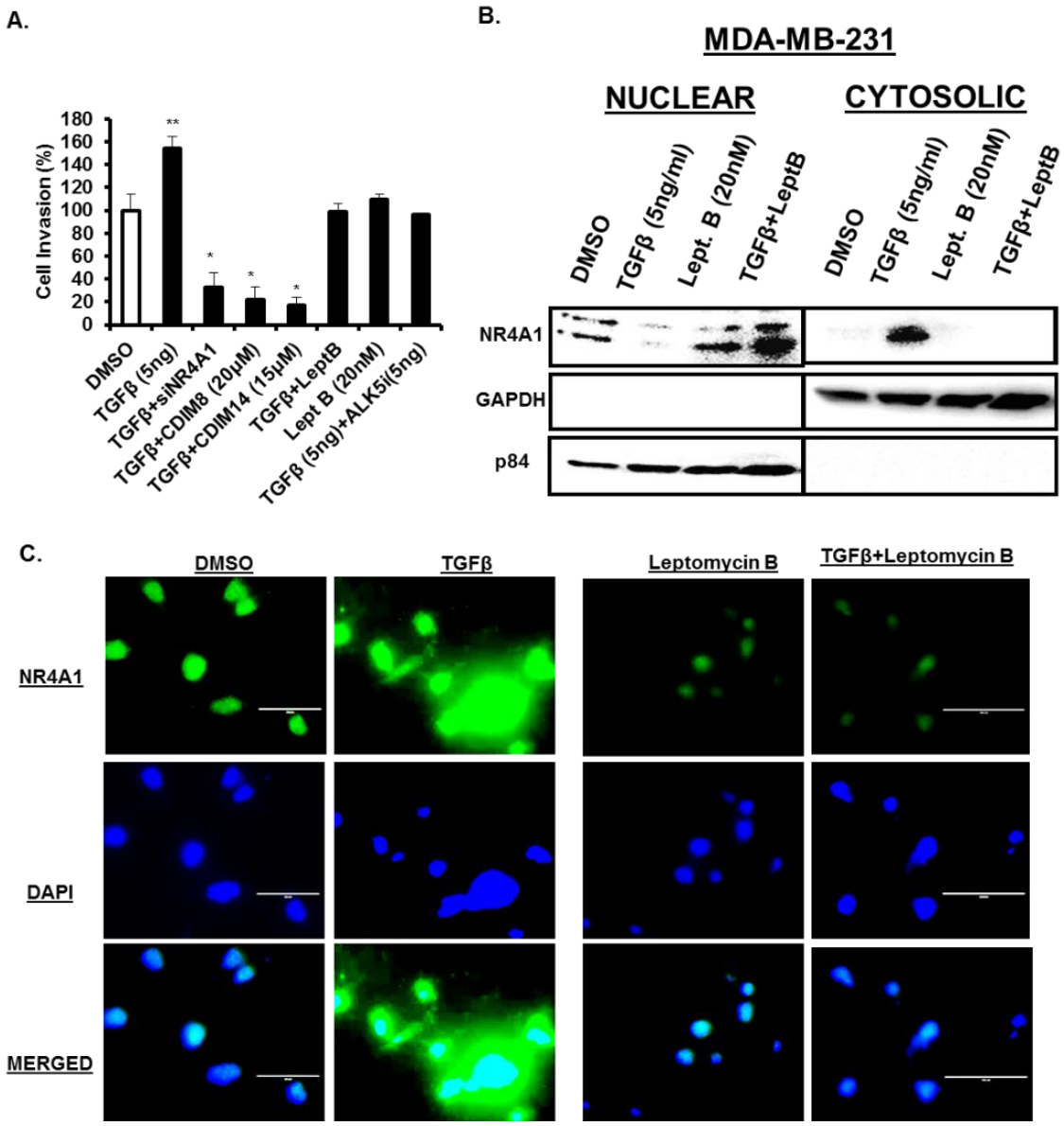

Figure 84. Role of NR4A1 on TGFβ-induced migration of MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with TGFβ alone for 5 hr or in combination with siNR4A1, DIM-CpPhOH and DIM-C-pPhCO2Me (24 hr treatment), LMB and ALK5i, and LMB (alone). Cell migration was determined in a Boyden chamber assay. (B) MDA-MB-231 cells were treated with DMSO, TGFβ and LMB (alone) and in combination for 5 hr. Nuclear and cytosolic extracts were analyzed by western blots using nuclear (p84) and cytosolic (GADPH) loading controls. (C) Cells were treated with DMSO, TGFβ, LMB alone and TGFβ in combination with LMB, DIM-C-pPhOH (CDIM8) or DIM-C-pPhCO2Me (CDIM14) for 4 hr, and whole cell lysates were analyzed for SMAD7 expression by western blot analysis. (D) Cells were treated with DMSO, 5 ng/ml TGFβ, LMB and LMB plus TGFβ for 5 hr and immunostained with both NR4A1 antibodies and DAPI as outlined in the Materials and Methods.

Since C-DIM/NR4A1 antagonists act through binding nuclear NR4A1, we examined the effects of short-term (4-h) treatment of MDA-MB-231 with DIM-C-pPhOH or DIM-C-pPhCO_2Me on TGF-β-induced migration. Like LMB, both compounds blocked TGF-β-induced migration (Fig. 85A), and this was accompanied by inhibition of TGF-β-induced nuclear export of NR4A1 (Fig. 85B) and paralleled results observed for LMB (Fig. 5A and B). The inhibitory effects observed after treatment with the C-DIM/NR4A1 ligands for 4 h were not due to decreased β1-integrin expression (Fig. 85C), suggesting that bound NR4A1 was resistant to TGF-β-induced nuclear export, and the factors that regulate nuclear export are currently being investigated. A previous report showed that TGF-β-induced NR4A1 interacts with axin 2 and other factors (e.g., E3 ligases Arkadia and RNF12) to form a polyubiquitination complex, and after treatment of MDA-MB-231 cells with TGF-β, LMB, C-DIMs, and their combinations, Western blot analysis of the cytosolic fraction immunoprecipitated with axin 2 antibodies gave a strong band for NR4A1 only in cells treated with TGF-β alone (Fig. 85D). In contrast, treatment with DIM-C-pPhOH, DIM-C-pPhCO_2Me, or LMB, which inhibit TGF-β-induced nuclear export of NR4A1, resulted in decreased intensities of cytosolic NR4A1 bands associated with the axin 2 antibody immunoprecipitates. The results demonstrate that NR4A1 plays an important role in breast cancer cell migration by regulation of β1-integrin (endogenous activity) and TGF-β-induced migration, which is dependent on NR4A1 nuclear export (Fig. 85E).

Figure 85.

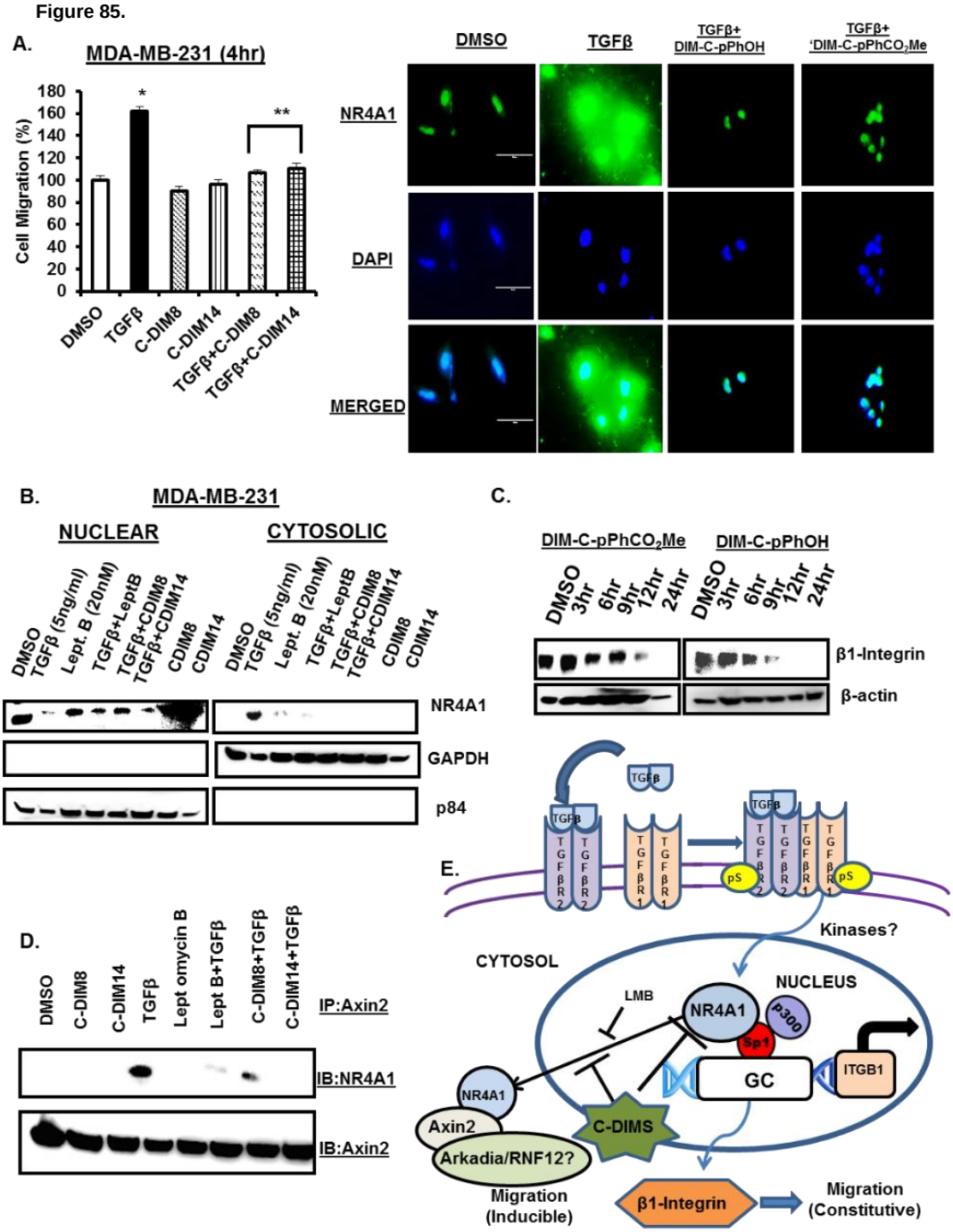

Figure 85. C-DIMs inhibit TGF-β-induced migration and NR4A1 nuclear export. (A) MDA-MB-231 cells were treated with TGFβ, DIM-C-pPhOH and DIM-CpPhCO2Me alone and TGFβ plus C-DIMs for 4 hr, and cell migration was determined in a Boyden Chamber assay and immunostaining (NR4A1) and DAPI staining was determined as outlined in Figure 5C. (B) Cells were treated as described in (Fig. 5B) and the cytosolic and nuclear extracts were further examined by western blot analyses. (C) MDA-MB-231 cells were treated with DIM-C-pPhCO2Me or DIM-C-pPhOH for different times and whole cell lysates were analyzed by western blots for β1-integrin expression. (D) Cells were treated as outlined in Figure 6B and whole cell lysates were immunoprecipitated with axin 2 antibodies and analyzed bywestern blots. (E) Schematic outline of the role of NR4A1 in constitutive and TGFβ-induced migration in breast cancer cells.

Discussion

The NR4A family of orphan nuclear receptors NR4A1, NR4A2, and NR4A3 were initially identified as stress-induced immediate early genes with a characteristic domain structure observed for nuclear receptors. NR4A receptors have both unique and overlapping functions, and there is increasing evidence that they play an important role in cellular homeostasis and diseases associated with metabolism, cardiovascular and neurological functions, inflammation, and the immune system (791,892,1014). Endogenous ligands for NR4A1 have not been identified; however, synthetic ligands that are structurally related to cytosporone B have been developed (906,911,941) and have potential clinical applications. For example, ethyl[2,3,4-trimethoxy-6-(*i*-octanoyl)phenyl]acetate is an NR4A1 ligand that acts as a receptor antagonist to decrease NR4A1-dependent hepatic gluconeogenesis and lower blood glucose levels in a rodent model for type 2 diabetes (941). NR4A1 is also overexpressed in solid tumors including both ER-positive and ER-negative breast tumors and is a negative prognostic factor for lung, colon, and breast cancer patients (822,895,1007).

Initial studies targeting NR4A1 for cancer chemotherapy showed that cell death observed in some cancer cell lines treated with several apoptosis agents was due to nuclear export of NR4A1 and the subsequent interactions of NR4A1 with bcl-2 to form a proapoptotic complex that disrupted mitochondria (905,938). The proapoptotic effects were also observed using peptides and paclitaxel that mimic NR4A1 interactions with bcl-2 (906,907). Studies in this laboratory have

identified C-DIMs as NR4A1 ligands that act as antagonists in cancer cell lines, and previous studies have demonstrated that C-DIM/NR4A1 antagonists inhibit growth and induce cell death through inactivation of nuclear NR4A1-dependent prooncogenic pathways in pancreatic, lung, colon, kidney, and breast cancer cell lines (382,788,791,803-806,812,822,892,904,1009-1014).

A recent report showed that high expression of NR4A1 in breast tumors correlated with decreased relapse-free survival, and this was linked to the role of NR4A1 in TGF-β and TGF-β/cytokine-induced migration/invasion and metastasis (822). Results of ongoing genomic and functional studies in several cancer cell lines identified β-*integrin* as a possible NR4A1-regulated promigration/invasion gene, and this correlated with previous *in vivo* studies showing that β1-integrin was important for metastasis of mammary tumors overexpressing the *erbB2* oncogene (846,862,1007,1008). Results in Fig.80, 81, and 83 demonstrate that knockdown of NR4A1 or treatment with the NR4A1 antagonists DIM-C-pPhOH and DIM-C-pPhCO$_2$Me decreased expression of β1-integrin protein and mRNA and β1-integrin-dependent responses in MCF7, MDA-MB-231, and SKBR3 cells and also inhibited migration of the latter two cell lines.

The mechanism of NR4A1 regulation of β-integrin in SKBR3 and MDA-MB-231 cells did not involve direct binding to *cis*-acting genomic sequences but through an indirect mechanism in which NR4A1/p300 act as a coregulatory complex to activate Sp1-regulated genes. The ChIP assays show that NR4A1, Sp1, and p300 interacted at the GC-rich region of the *β1-integrin* gene promoter

(Fig. 82), and knockdown of any one of these factors or treatment with C-DIM/NR4A1 antagonists resulted in decreased β1-integrin expression. These results are similar to those previously observed for NR4A1/p300/Sp1-mediated regulation of survivin in pancreatic cancer cells (804) and are consistent with other reports showing that other nuclear receptors also regulate expression of other Sp-dependent genes through NR4A1/Sp1 complexes (957-961). Previous studies show that knockdown or inhibition of β1-integrin in breast cancer cells results in the expression of β3-integrin, and this "integrin-switching" enhances TGF-β-induced metastasis, which presents a problem for applications of β1-integrin inhibitors in treatment of breast cancer. Like β1-integrin, the 5′-promoter region of the β3-integrin gene contains GC-rich sequences, and our results demonstrate that NR4A1 also regulates β3-integrin expression, and NR4A1 antagonists or NR4A1 knockdown decreases expression of both genes (Fig. 82). Thus, coregulation of β1- and β3-integrin by NR4A1 negates the "integrin-switching" phenomena and further demonstrates that the C-DIM/NR4A1 antagonists represent a novel therapeutic approach for inhibiting β1/β3-integrin-induced signaling and metastasis in breast cancer cells.

MDA-MB-231 and SKBR3 cells readily migrate in the absence of TGF-β or cytokine stimulus, and results of RNAi studies show that inhibition of cell migration by C-DIM/NR4A1 antagonists was observed only in cells expressing NR4A1 or β1-integrin (Fig. 83). Moreover, since the inhibitory effects of C-DIMs were similar in the presence or absence of the nuclear export inhibitor LMB (Fig. 83F), our

results indicate that constitutive migration of these cells was due to nuclear NR4A1-dependent regulation of β1-integrin. This is also supported by the observation that the TGF-β receptor inhibitor ALK5i inhibits TGF-β-induced migration but does not affect the high rate of constitutive migration of MDA-MB-231 cells (Fig. 84A). A recent study showed that NR4A1 was also required for TGF-β-induced migration of MDA-MB-231 and other cell lines, and this was due to interactions of NR4A1, axin 2, and E3 ligases which enhanced SMAD7 degradation, resulting in activation of the TGF-βR1 pathway (822).

We also observed that TGF-β induced NR4A1 expression and migration of MDA-MB-231 cells; however, the key essential element in this pathway was that TGF-β induced nuclear export of NR4A1 (Fig. 84B and D and 85C). Moreover, inhibition of nuclear export by the NR4A1 ligands (DIM-C-pPhOH or DIM-C-pPhcO_2Me) or LMB also blocked TGF-β-induced migration and enhanced SMAD7 expression (Fig. 84C). Previous studies on SMAD7 degradation in MDA-MB-231 cells used transfected FLAG-SMAD7, whereas in this study, we observed low to nondetectable SMAD7 expression in MDA-MBA-231 and SKBR3 cells. However, LMB, DIM-C-pPhOH, and DIM-C-pPhCO_2Me which prevent NR4A1 export also increased SMAD7 expression in cells cotreated with these compounds plus TGF-β (Fig. 84C), and this is consistent with a role for cytosolic NR4A1 in SMAD7 degradation as previously reported. Thus, TGF-β-induced migration of MDA-MB-231 cells is due to nuclear export of NR4A1, and the C-DIM/NR4A1 antagonist

blocks this pathway presumably by inhibiting factors/pathways required for nuclear export, and these factors/pathways are currently being investigated.

In summary, results of this study show that nuclear NR4A1 regulates β1-integrin expression in breast cancer cells, and C-DIM/NR4A1 antagonists inhibit expression of β1-integrin and β1-integrin-mediated responses, including cell migration, and the antagonists also inhibit NR4A1-regulated expression of β3-integrin. In contrast, TGF-β-induced migration of MDA-MB-231 cells requires nuclear export of NR4A1 which is inhibited not only by LMB but also by C-DIM/NR4A1 antagonists. Thus, constitutive migration and TGF-β-induced migration are dependent on nuclear and extranuclear NR4A1, respectively, and the C-DIM/NR4A1 antagonists inhibit both pathways by decreasing NR4A1-dependent expression of β1-integrin and by inhibition of TGF-β-induced nuclear export of NR4A1 (Fig. 85E). This study expands on the prooncogenic functions of NR4A1 and indicates that C-DIM compounds and other NR4A1 antagonists represent an important new class of mechanism-based anticancer drugs for treating patients with tumors overexpressing this receptor.

CHAPTER VI

SPECIFICITY PROTEIN (Sp) TRANSCRIPTION FACTORS Sp1, Sp3, AND Sp4 ARE NON-ONCOGENE ADDICTION GENES IN CANCER CELLS

Introduction

Activation or amplification of oncogenes play an important role in tumor formation, growth and metastasis, and multiple chemotherapies have been designed to target one or more oncogenes (987,1015). The addiction of cancer cells and tumors to oncogenes is due, in part, to their regulation of multiple growth-promoting, pro-survival and migration/invasion pathways that are the hallmarks of cancer (60). Mechanism-based antineoplastic agents, such as the tyrosine kinase inhibitor Gleevac/Imatinib that inhibits the oncogene BCR-ABL, have been successful; however, clinical applications of many other drugs targeting oncogenes including the tyrosine kinase inhibitors have had limited success due to multiple factors including the development of drug resistance (1016).

The proposed concept of non-oncogene addiction (NOA) by cancer cells includes "genes and pathways (are) essential to support the oncogenic phenotype of cancer cells but are not required to the same degree for the viability of normal cells" (987). For example, ATM-deficient cells exhibit decreased apoptosis and exhibit NOA to DNA-dependent protein kinases and, drugs that inhibit these kinases are highly effective for treating ATM-deficient lymphomas (1016).

Specificity protein (Sp) transcription factors (TFs) Sp1, Sp3 and Sp4 are members of the Sp/Krüppel-like family (KLF), and results from Sp knockout mouse models demonstrate the importance of Sp genes to embryonic growth and early development (reviewed in 1017). However, expression of Sp1 in humans and rodents decreases with age (988-990). Moreover, several studies report that high expression of Sp1 and, in some cases, Sp3 in tumor vs. non-tumor tissue are negative prognostic factors for patients with pancreatic, glioma, colon, gastric, head and neck, prostate, lung and breast cancers (991,994,995,1018-1023). Studies in cancer cell lines show that Sp1, Sp3 and Sp4 are highly expressed, and RNA interference (RNAi) studies indicate that Sp transcription factors regulate genes associated with cell proliferation, survival and migration/invasion (reviewed in 996). Although Sp1, Sp3 and Sp4 have similar modular structures and bind GC-rich promoter sequences, these transcription factors also exhibit unique properties including the number of isoforms and DNA binding characteristics (711,1024,1025). Moreover, since Sp1 regulates expression of both pro-oncogenic and tumor suppressor-like genes, it has been suggested that "a more complete understanding of the function of Sp1 in cancer is required to validate its potential as a therapeutic target" (711).

Although knockdown of Sp1 by RNA interference (RNAi) in cancer cell lines inhibits cell growth, survival and migration/invasion (648,976,982,1026,1027), a systematic comparative analysis of the functional and genomic effects of Sp1, Sp3 and Sp4 in cancer cells has not been reported. In this study, we show that

knockdown of Sp1, Sp3 and Sp4 by RNAi in SKBR3 and MDA-MB-231 breast, A549 lung, SW480 colon, 786-O kidney, and Panc1, L3.6pL and MiaPaCa2 pancreatic cell lines results in inhibition of cell growth, decreased survival, and inhibition of migration/invasion. Using Panc1 cells as a model, a causal Ingenuity Pathway Analysis (IPA) of changes in gene expression after knockdown of Sp1, Sp3 and Sp4 strongly correlated with observed changes in functional responses. Thus, the oncogenic-like activity of Sp transcription factors and Sp-regulated genes coupled with their overexpression in tumor vs. non-tumor tissue indicates that Sp1, Sp3 and Sp4 are NOA genes that are "attractive drug targets" (987).

Materials and Methods

Cell lines and antibodies

Breast cancer (SKBR3, MDA-MB-231), kidney cancer (786-0), colorectal cancer (SW480), lung cancer (A549), and pancreatic cancer (PANC1,l3.6pL, Miapaca2) cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained 37˚C in the presence of 5% CO2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic or RPMI-1640 Medium with 10% fetal bovine serum and antibiotic. B-actin antibody Dulbecco's Modified Eagle's Medium, and RPMI-1640 Medium, and 36% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Hematoxylin was purchased from Vector Laboratories (Burlingame, CA).

Sp1 antibody from Millipore (Temecula, CA); Sp3, Sp4, EGFR, bcl2 antibodies from Santa Cruz Biotech (Santa Cruz, CA); survivin antibody from Cell Signaling technologies (Danvers, MA); VEGF antibody from GeneTex (Irvine, CA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA). Cells were visualized as described previously (711).

Cell proliferation assay and Annexin V staining

Cell proliferation assays were carried out as described previously (648,976,1027,1028)) and changes in cell number were determined by Coulter Z1 cell counter. Annexin V staining used the Vybrant apoptosis kit according to the manufacturer's protocol (711).

Boyden Chamber Assay

SKBR3, MDA-MB-231, 786-0, SW480, A549, PANC1, l3.6PL, and Miapaca2 cancer cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. Cells were seeded and subsequently treated with varying concentration of panobinostat or vorinostat for 24 hours (+/- GSH 3 h prior to treatment) or with 100 nm of siSp1, siSp3, siSp4 for 48 hours. Cells were trypsinized, counted then placed in 12-well 8.0µm pore ThinCerts from Greiner bio-one (Monroe , NC) allowed to migrate for 24 hr, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted as described (711).

Western blot analysis

SKBR3, MDA-MB-231, 786-0, SW480, A549, PANC1, l3.6PL, and Miapaca2 cancer cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. Cells were transfected with 100 nm of siSp1, siSp3, siSp4 for 72 hours. Cells were analyzed by western blot as described previously (648,976,1027-1029)).

Small interfering RNA interference assay

SiRNA experiments were conducted as described previously() The siRNA complexes used in the study are as follows: siGL2-5': CGU ACG CGG AAU ACU UCG A; siSp1:SASI_Hs02_00333289[1], SASI_Hs01_00140198[2], SASI_Hs01_00070995[3]; siSp3: SASI_Hs01_00211941[1], SASI_Hs01_00211942[2], SASI_Hs01_00211943[3]; siSp4: SASI_Hs01_00114420[1], SASI_Hs01_00114421[2], SASI_Hs01_00114420[3]

Xenograft Studies

Female athymic nude mice 4-6 old were purchased as previously described (28). L3.6pL cells in culture were transfected with 100 nM of siCtl (7 mice), siSp1 (7 mice), or siSp1,3,4 (7 mice). After 48hr 1.0 x106 cells were suspended in Matrigel (1:1 ratio) and injected into the right flank of athymic nude mice. Tumor volumes, tumor weights, and tumor lysates were determined and analyzed as previously described (711).

Microarray and IPA analysis

After knockdown by RNAi total RNA was extracted using a mirVanaTM miRNA Isolation Labeling Kit (Ambion, Austin, TX) and used for microarray analysis with a HumanHT-12 v4 expression beadchip (Illumina, San Diego, CA) according to the manufactures' protocol. Microarray data were normalized and results from replicate (3X) experiments were used to identify differentially expressed genes with a ≥ 1.5-fold change. Function and pathways analysis of Sp-regulated genes was determined using Ingenuity Pathways Analysis (IPA) database (Invitrogen, Carlsbad, CA).

Statistical analysis

Statistical significance of differences between the treatment groups was determined as previously described (711).

Results

Figure 86.

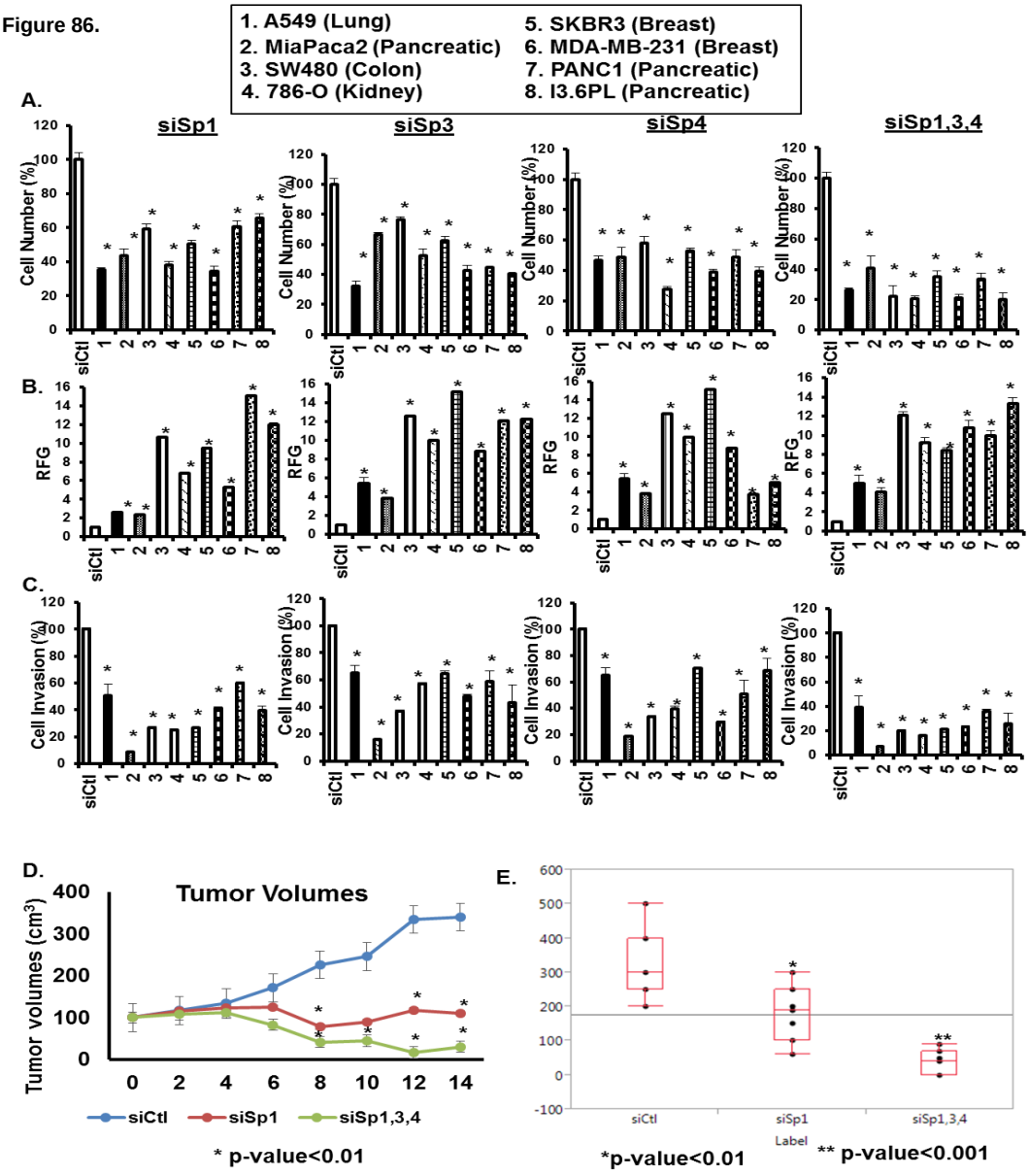

Figure 86. Functional effects of Sp1, Sp3 and Sp4 knockdown in cancer cells. Effects of knockdown in A549, MiaPaCa2, SW480, 786-O, SKBR3, MDA-MB-231, Panc1 and L3.6pL cancer cell lines. Cells were transfected with siSp1, siSp3 and siSp4 and effects on cell proliferation (A), Annexin V staining (B) and invasion in a Boyden chamber assay (C) were determined as described in the Materials and Methods. Results are expressed as means ± SE for at least 3 biological replicates for each determination, and significant (p<0.05) changes compared to cells transfected with a nonspecific oligonucleotide (siCtl) are indicated (*). (D) Knockdown of Sp1, Sp3 and Sp4 (combined) or Sp1 alone in L3.6pL cells were used in xenografts experiments and changes in tumor volumes/weights were determined essentially as described (22-25). Significant changes (p < 0.05) after Sp knockdown are indicated (*).

Knockdown of Sp transcription factors in cancer cell lines: functional effects.

The functional and genomic effects of Sp1, Sp3 and Sp4 and their possible roles as NOA genes were investigated by RNAi in several different cancer cell lines. Multiple oligonucleotides for Sp1, Sp3 and Sp4 have previously been used for studying Sp-regulated gene expression and functional responses (Suppl. Fig. A-9) (648,976,1027-1029), and a single representative oligonucleotide was used for this study. Figure 1 summarizes the effects of knockdown of Sp1, Sp3 and Sp4 in A549, MiaPaCa2, L3.6pL, Panc1, SW480, 786-O, SKBR3 and MDA-MBA-231 cancer cell lines. Decreased expression of Sp1, Sp3 and Sp4 resulted in significant inhibition of cancer cell proliferation (Fig. 86A), induction of Annexin V (a marker of apoptosis) (Fig. 86B), and inhibition of cancer cell invasion in a Boyden Chamber assay (Fig. 86C) and knockdown of all three genes (siSp1,3,4) enhanced the observed responses. The magnitude of the effects showed some variability and was dependent on the individual Sp protein and cell context. Most previous functional studies have focused on Sp1; however, results illustrated in Figure 1 clearly demonstrate that both Sp3 and Sp4 also contribute to the growth, survival and migration/invasion of the eight cancer cells lines. In a parallel experiment, combined knockdown of Sp1, Sp3 and Sp4 or individual knockdown of Sp1 in L3.6pL cells used in an athymic nude mouse xenograft model showed that loss of Sp TFs resulted in a significant inhibition of tumor growth (Fig. 86D).

Table 1

siSp	Categories	Diseases or Functions Annotation	p-Value	Predicted Activation State	Activation z-Score	# of Molecules
siSp1	Cell death and survival	Cell death	1.11E-43	Increased	2.821	749
	Cellular growth and proliferation	Proliferation of cells	2.66E-39	Decreased	-3.240	788
	Cellular movement	Migration of tumor cell lines	6.40E-11	Decreased	-2.063	150
siSp3	Cell death and survival	Cell death	1.19E-32	Increased	2.526	975
	Cellular growth and proliferation	Proliferation of cells	8.07E-27	Decreased	-5.410	1024
	Cellular movement	Migration of tumor cell lines	2.54E-08	Decreased	-6.346	190
siSp4	Cell death and survival	Cell death	2.62E-34	Increased	3.809	995
	Cellular growth and proliferation	Proliferation of cells	4.41E-28	Decreased	-6.222	1044
	Cellular movement	Migration of tumor cell lines	2.42E-09	Decreased	-6.411	197

Table 1: Causal IPA analysis of Sp knockdown. Causal IPA analysis gene functions of Sp knockdown by RNAi in Panc1 cells.

Figure 87.

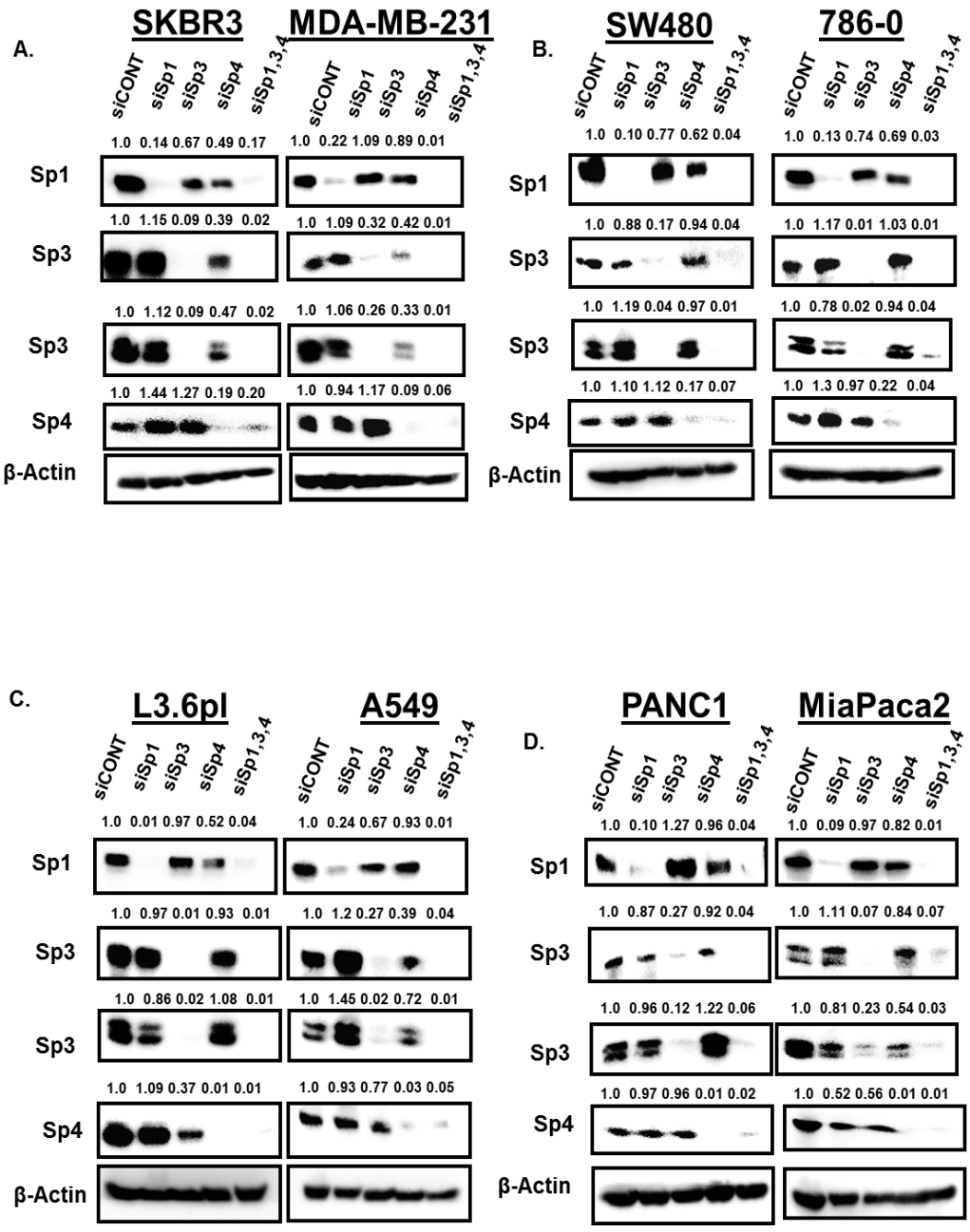

Figure 87. Knockdown of Sp TFs by RNAi. (A) SKBR3 and MDA-MBA-231, (B) SW480 and 786-O, (C) L3.6PL and A549, and (D) Panc1 and MiaPaCa2 cells were transfected with siSp1, siSp3 and siSp4, and whole cell lysates were analyzed by Western blots as outlined in the Materials and Methods.

Knockdown of Sp1, Sp3 and Sp4 in cancer cell lines: effects on Sp TFs and Sp-regulated gene products

The individual effects of Sp1, Sp3 and Sp4 knockdown could be indirect since the three genes contain GC-rich promoters and they could be self-regulatory (1027-1029) and this was further investigated. Western blot analysis of expression of Sp TFs after transfection of the cancer cell lines with siControl (siCtl, non-specific oligonucleotide) or oligonucleotides targeting Sp1 (siSp1), Sp3 (siSp3), Sp4 (siSp4), or their combination (siSp1,3,4) showed that Sp1, Sp3 and Sp4 proteins were highly expressed in the eight cancer cell lines (Fig. 87). Knockdown of Sp1, Sp3 and Sp4 was specific for the individual proteins in SW480, 786-O, A549, Panc1 and MiaPaCa2 cells. In contrast, siSp3 decreased expression of Sp1 (SKBR3) and Sp4 (L3.6pL) proteins and siSp4 decreased Sp1 (SKBR3) and Sp3 (SKBR3 and MDA-MB-231) proteins. These results demonstrate that autoregulation of Sp1, Sp3 and Sp4 was minimal among the eight cancer cell lines and primarily involved Sp3 and Sp4 and their regulation of each other or of Sp1. Sp TFs regulate expression of several pro-oncogenic factors including vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), survivin and bcl2 (996). In this study, we examined the effects of Sp knockdown on expression of these genes and induction of the apoptotic marker cleaved PARP in the cancer cell lines. Transfection of siSp1, siSp3, siSp4 and siSp1,3,4 induced PARP cleavage in all eight cell lines (Fig. 88) and this complemented the induction of Annexin V staining (Fig. 86B) observed

after the same treatments. EGFR, VEGF, bcl-2 and survivin were expressed in the eight cancer cell lines, and siSp1, siSp3 and siSp4 downregulated these gene products but some variability that was si-oligonucleotide-, gene- and cell context-dependent was observed.

Figure 88.

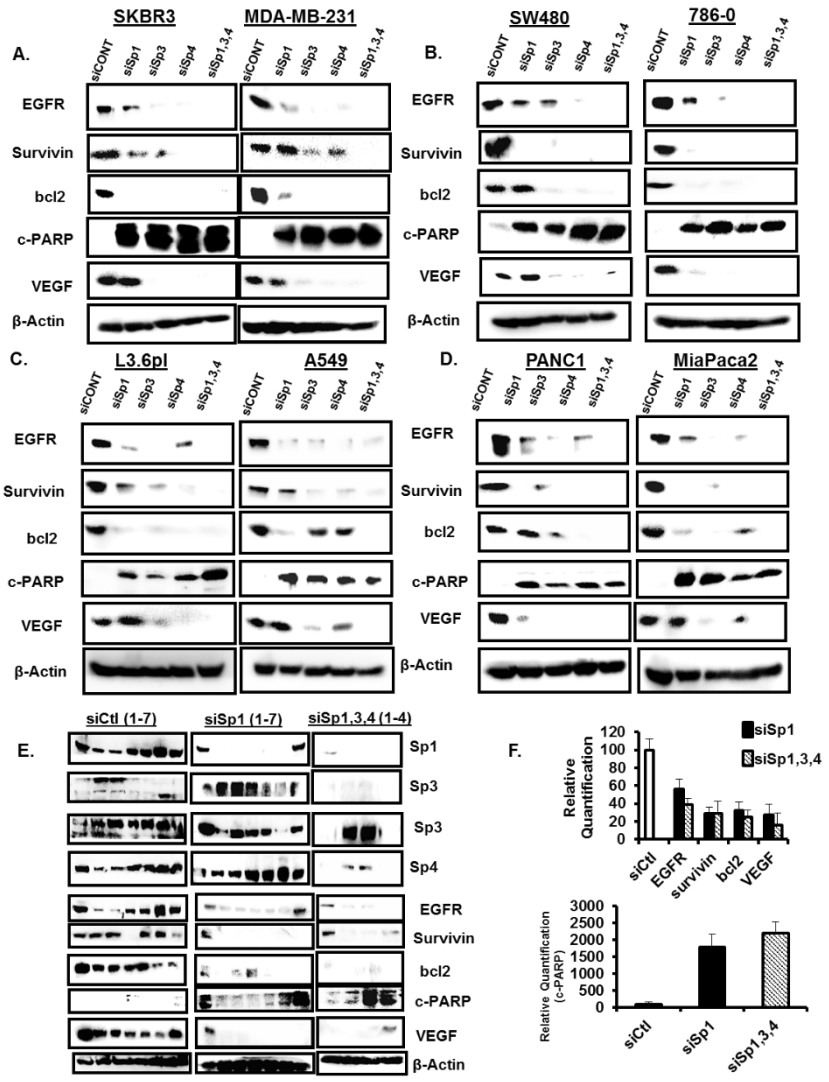

Figure 88. Knockdown of Sp TFs decreases expression of Sp-regulated gene products. Cell lines (A – D) and L3.6pl xenogratfs (E) were transfected as described in Figure 2 and these same lysates were analyzed for expression of Sp-regulated gene products as outlined in the Materials and Methods. Tumor lysates were normalized to β-actin (F).

Figure 89.

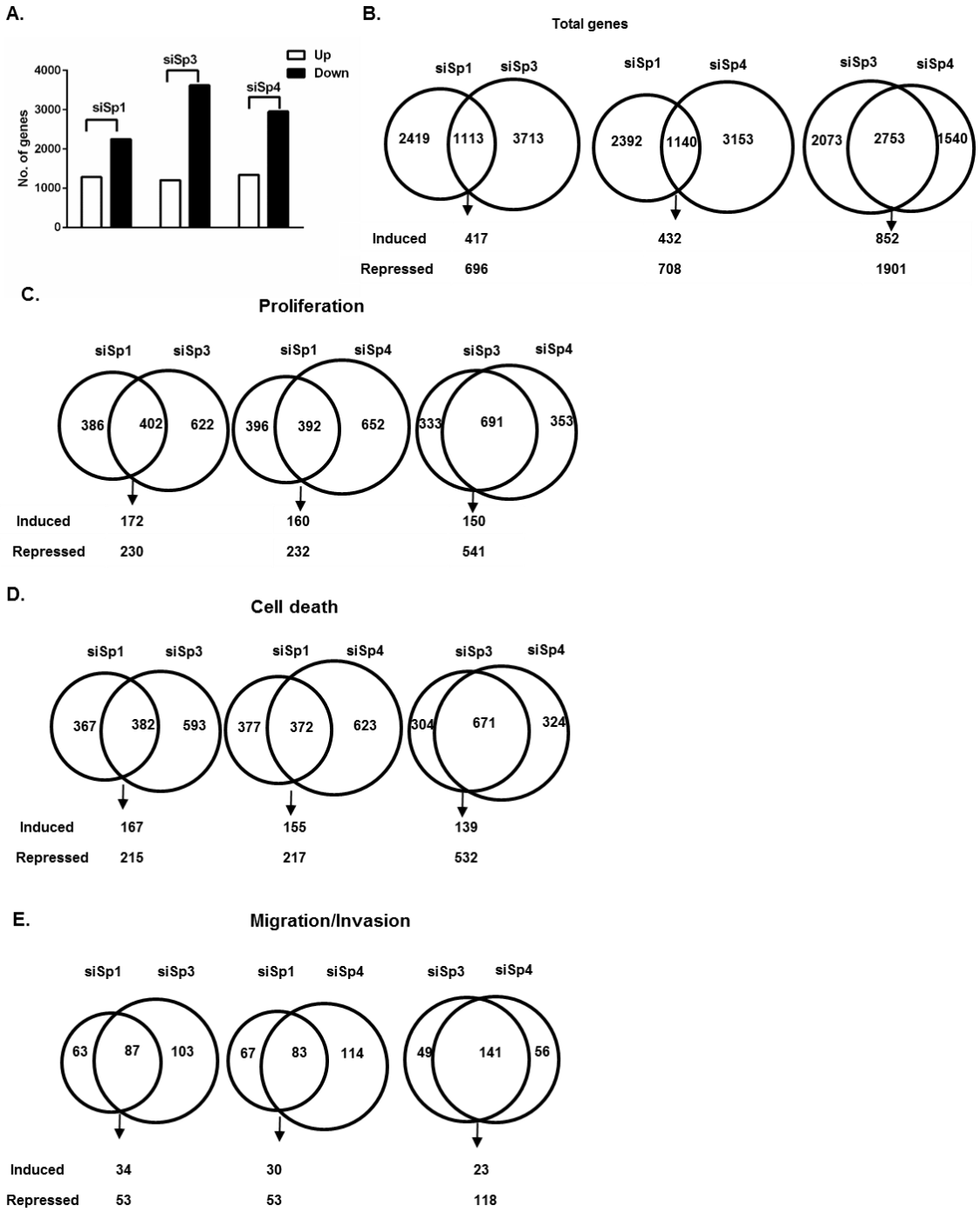

Figure 89. Analysis of changes in gene expression after knockdown of Sp1, Sp3 and Sp4 in Panc1 cells. (A) Panc1 cells were transfected with siSp1, siSp3 or siSp4, and changes in gene expression were determined using Human HT-12 V4 expression bead chip arrays. The overlap of total genes (B) and proliferation (C), survival (D) and invasion (E) genes coregulated by Sp1/Sp3, Sp1, Sp4 and Sp3/Sp4 in Panc1 cells after RNAi was determined by IPA.

Analysis of gene expression changes in Panc1 cells after knockdown of Sp1, Sp3 and Sp4

Results of RNAi studies show that Sp1, Sp3 and Sp4 exhibited pro-oncogenic activity and regulated pro-oncogenic factors (Figs. 86 and 88). This was further investigated in gene array studies using Panc1 cells as a model. Transfection of Panc1 cells with siSp1, siSp3 and siSp4 and analysis of gene expression using arrays resulted in inhibition or induction of 3,532, 4,826 and 4,293 genes, respectively (Fig. 89A). After knockdown of Sp1, Sp3 and Sp4, Venn diagrams show considerable overlap of genes commonly regulated by Sp1:Sp3 (1,113); Sp1:Sp4 (1,114) and Sp3:Sp4 (2,753) with the most pronounced gene overlap observed for Sp3 and Sp4 (Fig. 89B). IPA was used to investigate common and differentially expressed genes after knockdown of Sp1, Sp3 and Sp4 associated with cell proliferation, survival and migration/invasion and there were significant changes in total gene expression associated with cell proliferation (788, 1,204 and 1,044 genes, respectively), survival (759, 975 and 995 genes, respectively) and migration/invasion (150, 190 and 197 genes, respectively) (Fig. 89C-89E). Venn diagrams also showed that there was also a considerable overlap of common genes coregulated by Sp1:Sp3, Sp1:Sp4 and Sp3:Sp4 associated with cell proliferation (Fig. 89C), survival (Fig. 89D) and migration/invasion (Fig. 89E). For example, after knockdown of Sp3 and Sp4 by RNAi, there was a 60-70% overlap of genes associated with Panc1 cell

proliferation, survival and migration/invasion and this correlated with their common regulation of total genes (Fig. 89B).

Examination of the changes in gene expression after RNAi showed that there were Sp1-, Sp3- and Sp4-regulated genes that correlated or inversely correlated with the observed functional responses induced by knockdown of Sp TFs (Fig. 86). This was confirmed by real time PCR analysis (Fig. 90) showing that one or more Sp TFs decreased expression of the tumor promoting genes ribonucleotide reductase M2 (RRM2) and Aurora kinase A (AURKA) and increases expression of the tumor suppressor-like genes such as thioredoxin-interacting protein (TXNIP) and the polycomb CBX7 genes (1029-1032). However, knockdown of one or more Sp TFs also increased expression of genes such as heme oxygenase 1 (HMOX1) and interferon-stimulated gene 15 (ISG15) that promote carcinogenesis and decreased expression of caspase 3 (CASP3) and Sprouty2 (SPRY2) that inhibit pancreatic tumorigenesis (1034-1037). These results are consistent with the array data showing that Sp TFs regulate genes that both correlate and inversely correlate with the results of functional studies (Suppl. Tables B1-B3).

Causal analysis using IPA is a quantitative approach that integrates all of the changes in expression of genes and pathways in large data sets to predict biologic function (1038). Table 1 summarizes the analysis of the total changes in gene expression after knockdown of Sp1, Sp3 and Sp4 in Panc1 cells. The low p-values and activation score values (>2 or <-2) obtained from this analysis

strongly predicted that Sp1, Sp3 and Sp4 were associated with Panc1 cell proliferation, survival and migration/invasion and were consistent with the functional results illustrated in Figure 86. These functional and genomic data coupled with the high expression of Sp transcription factors in tumor vs. non-tumor tissue suggests that Sp1, Sp3 and Sp4 are NOA genes and attractive drug targets.

Figure 90.

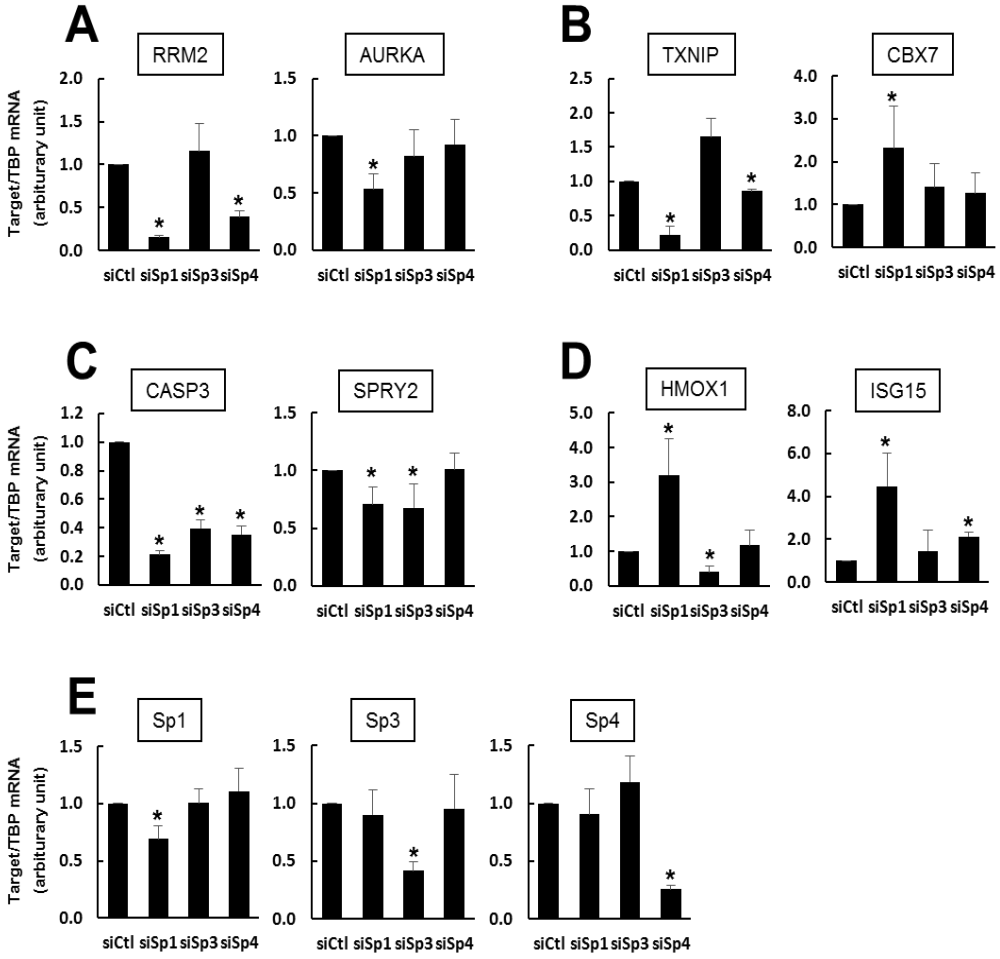

Figure 90. Changes in expression of specific genes after Sp knockdown in Panc1 cells. Panc1 cells were transfected with siSp1, siSp3 or siSp4, and real time PCR analysis was used to determine changes in expression of (A) RRM2 and AURK, (B) TXNIP and CBX7, (C) CASP3 and SPRY2, (D) HMOX1 and ISG15, and (E) Sp1, Sp3 and Sp4. Results are expressed as means ± SE for at least 3 replicates for each treatment group, and significantly ($p<0.05$) decreased changes in gene expression are indicated (*).

Discussion

The concept of NOA highlights the fact that the cancer genotype and hallmarks of cancer are maintained by both oncogenes and NOA genes which are also important targets for mechanism-based anticancer agonists (60,987,1015). Among Sp/KLF transcription factors, Sp1 has been most extensively investigated and fulfills many of the criteria for an NOA gene. Sp1 levels decrease with age in rodents and humans (988-990) and several studies show that Sp1 levels are high in tumor vs. non-tumor tissue (991,994,995,1018-1023). The differential expression of Sp1 has also been observed in human fibroblasts where carcinogen- or oncogene-induced transformation resulted in an 8- to 18-fold increase in Sp1 levels (998). Moreover, in xenograft experiments, the loss of Sp1 in fibrosarcoma cells decreased their ability to form tumors (998) and the role of Sp1 in tumor growth, survival and migration/invasion has been confirmed in other reports (648,976,982,996,1026).

Our results in multiple cancer cell lines clearly demonstrate that not only Sp1 but also Sp3 and Sp4 play a role in cancer cell growth, survival and migration/invasion (Fig. 86) and regulate expression of gene products (Fig. 88) consistent with these observations. Moreover, transfection of Panc1 cells with siSp1, siSp3 or siSp4 and IPA of changes in gene expression by arrays showed that all three transcription factors regulated genes that enhance cell proliferation, survival and migration/invasion (Suppl. Tables B1-B3). Despite overlap in their regulation of common genes (Fig. 89), RNAi studies on functional effects of Sp

TFs (Fig. 86) showed that individual loss of Sp1, Sp3 or Sp4 was sufficient to decrease growth, survival and invasion and compensation by the other two Sp genes was not observed, suggesting unique gene regulatory functions for these transcription factors.

As recently pointed out (711), Sp1 regulates expression of genes that both enhance and inhibit carcinogenesis as indicated in Supplemental Tables S1-S3 and Figure 5 and this is a possible cautionary consideration for clinical development of anticancer drugs that specifically target Sp proteins. However, causal IPA approaches which weigh contributions of individual genes to various networks/pathways (Table 1) showed that examination of all genes associated with cancer cell proliferation, survival and migration/invasion after Sp knockdown strongly correlated with the observed functional responses (Fig. 86).

Previous studies have reported differences in the prognostic value of Sp1 overexpression in breast and lung cancer patients and also differences in the pro- and anti-carcinogenic role of Sp1 in MDA-MB-231 breast and A549 lung cancer cell lines (1020,1039-1043). Using an RNAi approach (Fig. 86), our results show that not only Sp1 but also Sp3 and Sp4 exhibit pro-oncogenic activities in MDA-MB-231 and A549 cells. Some of the differences between studies may be due to the methods used to modulate Sp expression since overexpression of Sp1 and Sp3 in some cancer cell lines induces apoptosis and inhibits growth (1044-1047). It is possible that overexpressing Sp1, Sp3 and Sp4 may not always be ideal for probing the "constitutive" functions of these transcription factors since high

intracellular levels of Sp1, Sp3 and Sp4 may activate expression of genes with GC-rich promoters that are not normally expressed, and this is currently being investigated.

In summary, this study indicates that Sp1, Sp3 and Sp4 are NOA genes that are highly expressed in tumor vs. non-tumor tissue and regulate expression of pro-oncogenic factors that contribute to cancer cell growth, survival and migration/invasion. Several different classes of antineoplastic agents that target Sp transcription factors have been identified and these include natural products and their derivatives, metformin, non-steroidal anti-inflammatory drugs, and ROS-inducing anticancer agents, including isothiocyanates, piperlongumine and arsenic trioxide (1020,1039-1043). Moreover, drugs, such as ascorbate, tolfenamic acid and betulinic acid that downregulate Sp proteins, are highly effective in drug combinations for inhibiting tumor growth in laboratory animal studies (1048-1050). Important advantages for development and clinical applications of anticancer agents that target Sp1, Sp3 and Sp4 include: (a) these compounds decrease Sp-regulated genes such as EGFR, VEGF, cMET and other tyrosine kinases that are themselves individual drug targets; and (b) these agents also decrease expression of drug resistance genes (survivin, MDR1) (996) and are ideal for drug combination therapies. The choice of a specific drug for targeting Sp TFs will be tumor-specific and dependent on pharmacokinetics and efficient delivery of the agent to the tumor site.

CHAPTER VII

THE NUCLEAR ORPHAN RECEPTOR NR4A1 REGULATES β1-INTEGRIN EXPRESSION IN PANCREATIC AND COLON CANCER CELLS AND CAN BE TARGETED BY NR4A1 ANTAGONISTS

Introduction

The nuclear receptor 4A (NR4A) subfamily of orphan receptors including NR4A1 (Nur77/TR3), NR4A2 (Nurr1) and NR4A3 (Nor1) are stress-inducible immediate early genes that play both unique and overlapping roles in metabolic, cardiovascular, neurological and immune functions, inflammation, and cancer (892,1014)). Although endogenous ligands for NR4A receptors have not been identified, there is evidence that ligands for these receptors acting as agonists or antagonists are emerging as a novel class of therapeutics (788,808,905,1014). For example, Wu and coworkers have characterized cytosporone and related synthetic analogs as NR4A1 ligands (817,910,911,941,942) and have shown that one of their analogs inhibits endotoxin-induced sepsis in RAW 264.7 cells and *in vivo* (817). The mechanism of this NR4A1-dependent antiinflammatory response is due to ligand-induced dissociation of p38-NR4A1 interactions and restoration of the p65-NR4A1 complex resulting in inhibition of NFκB signaling (817).

In double knockout animals, the loss of both NR4A1 and NR4A3 results in the rapid development of acute myeloid leukemia (AML) in mice (1051) indicating

a tumor suppressor-like role for these receptors. In contrast, NR4A1 is overexpressed in many solid tumors and their derived cell lines, and in breast, colon and lung tumors overexpression of NR4A1 is a negative prognostic factor (803,804,822,895,954,1052,1053). Moreover, knockdown or overexpression of NR4A1 shows that this receptor is pro-oncogenic and regulates one or more of cell proliferation, survival and migration/invasion in lung, melanoma, lymphoma, kidney, pancreatic, colon, cervical, ovarian and gastric cancer cells (805,806,812,822,895,898,900,901,904,1053-1056).

Studies in this laboratory have identified 1,1-bis(3'-indolyl)-1-(substituted phenyl)methane (C-DIM) analogs as NR4A1 ligands that act as nuclear receptor antagonists (803-806,812,904). RNAseq or array analysis have shown that treatment with specific C-DIM/NR4A1 antagonists results in both induced and repressed gene expression which contribute to the NR4A1-regulated pro-oncogenic pathways. For example, in both liver and colon cancer cells, treatment with the NR4A1 antagonist 1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH) induces reactive oxygen species (ROS) through downregulation of isocitrate dehydrogenase 1 (IDH1) and thioredoxin domain containing 5 (TXNDC5) and downregulates survivin and other specificity protein (Sp)-regulated genes containing GC-rich promoters (803-806,812,904). Ligand-dependent inactivation of NR4A1 activates p53 and induces ROS which induce sestrin 2 and activation of AMPKα resulting in inhibition of mTOR (803,812,904). All of these pro-oncogenic NR4A1-regulated genes/pathways and functions are blocked by

C-DIM/NR4A1 antagonists in breast, pancreatic, colon, kidney and lung cancer cell lines (803-806,812,904).

Recent studies have identified β1-integrin as an NR4A1-regulated gene in breast cancer cells and the NR4A1 antagonist DIM-C-pPhOH decreases β1-integrin expression and also inhibits β1-integrin-dependent cell migration (805). Since β1-integrin is a negative prognostic factor and induces migration/invasion and metastasis in both pancreatic and colon cancer cells (1056-1067), this study investigated the role of NR4A1 in regulating β1-integrin expression and subsequent inhibition of β1-integrin-dependent migration/invasion by C-DIM/NR4A1 antagonists. The results confirm that C-DIM/NR4A1 antagonists target NR4A1 and inhibit invasion of pancreatic and colon cancer cells, demonstrating that these compounds represent a novel class of mechanism-based drugs for pancreatic and colon cancer chemotherapy.

Materials and Methods

Cell lines and antibodies

Pancreatic cancer (Panc1 and MiaPaCa2) and colon cancer (RKO, SW480) cell lines were purchased from American Type Culture Collection (Manassas, VA) and were authenticated on April 29, 2016 by Biosynthesis (Lewisville, TX). L3.6pL cells were kindly provided by Dr. I.J. Fidler (University of Texas MD Anderson Cancer Center, Houston, TX). Cells were maintained 37°C

in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic. NR4A1 antibody was purchased from Novus Biologicals (Littleton, CO). Sp1 antibody and glutathione (GSH) reduced free acid were purchased from Millipore (Temecula, CA); β1-integrin, p300, Sp3 and Sp4 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). β-Actin antibody, Dulbecco's Modified Eagle's Medium, and 36% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Hematoxylin was purchased from Vector Laboratories (Burlingame, CA). FAK, p-FAK, α5-integrin antibodies and leptomycin were purchased from Cell Signaling Technologies (Manassas, VA)

Cell adhesion assay

Panc1, L3.6pL, MiaPaCa2, RKO and SW480 cancer cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hr. Cells were seeded and subsequently treated with varying concentrations of DIM-C-pPhOH or DIM-C-pPhCO_2Me for 24 hr or with 100 nm of siβ1-integrin or siNR4A1 for 48 hr. Cells were trypsinized, counted, then placed for 90 min on BD BioCoat Human Fibronectin Cellware 24-well plates (Bedford , MA); medium was then aspirated, wells gently washed with PBS, and stained with 0.5% Crystal Violet Stain. Cells were then counted for adhesion to fibronectin. Wells coated with BSA and poly-L-lysine were used as negative controls.

Boyden chamber assay

Panc1, L3.6pL, MiaPaCa2, RKO and SW480 cancer cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hr. Cells were seeded and subsequently treated with varying concentrations of DIM-C-pPhOH or DIM-C-pPhCO$_2$Me for 24 hr or with 100 nm of siβ1-integrin, siNR4A1, siSp1, or sip300 for 48 hr. Cells then treated with varying concentrations of DIM-C-pPhOH or DIM-C-pPhCO$_2$Me for 24 hr after transfection. Cells were trypsinized, counted then placed in 24-well 8.0 μm pore ThinCerts from BD Biosciences (Bedford, MA) allowed to migrate for 24 hr, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted as described (812,904,1056).

RT PCR

RNA was isolated using Zymo Research *Quick-RNA* MiniPrep kit (Irvine, CA). Quantification of mRNA (β1-integrin) was performed using Bio-Rad iTaq Universal SYBER Green 1-Step Kit (Richmond, CA) using the manufacturer's protocol with real-time PCR. TATA Binding Protein (TBP) mRNA was used as a control to determine relative mRNA expression.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif,

Carlsbad, CA) according to the manufacturer's protocol. Panc1, L3.6pL, MiaPaCa2, RKO and SW480 cancer cells were treated with DMSO, DIM-C-pPhOH (15, 20 μM), or DIM-C-pPhCO$_2$Me (15, 20 μM) for 24 hr. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (~200 to 1,500 bp). The sonicated chromatin was immunoprecipitated with normal IgG, p300 (Santa Cruz), Sp1 (Abcam), NR4A1 (Novus Biologicals), Sp3, Sp4 (Santa Cruz), or RNA polymerase II (pol II; Active Motif) antibodies and protein A-conjugated magnetic beads at 4°C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. DNA was prepared by proteinase K digestion followed by PCR amplification. The primers for detection of the β1-integrin promoter region were 5'- TCACCACCCTTCGTGACAC -3' (sense) and 5'-GAGATCCTGCATCTCGGAAG-3' (antisense). PCR products were resolved on a 2% agarose gel in the presence of RGB-4103 GelRed Nucleic Acid Stain.

Western blot analysis

Panc1, L3.6pL, MiaPaCa2, RKO and SW480 cancer cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed

to attach for 24 hr. Cells were transfected with 100 nm of siβ1-integrin, siNR4A1, siSp1, siSp3, siSp4 (or in combination), or sip300 for 72 hr or treated with C-DIM/NR4A1 antagonists. Cells were analyzed by western blot as described previously (812,904,1056). Bands were also quantitated relative to β-actin with the DMSO/siCtl set at 1.0. Low intensity bands (>90% decreased compared to the control) were assigned a value of <0.01.

Small interfering RNA interference assay

SiRNA experiments were conducted as described previously (812,904,1056). The siRNA complexes used in the study were as follows: siGL2-5': CGU ACG CGG AAU ACU UCG A; siNR4A1: SASI_Hs02_00333289[1], SASI_Hs02_00333290[2]; siβ1-integrin: SASI_Hs02_00333437[1], SASI_Hs01_00159474; siSp1:SASI_Hs02_003; sip300: SASI_Hs01_00052818; siSp3, SASI_Hs01_00211941; siSp4, SASI_Hs01_00114420.

Statistical analysis

Statistical significance of differences between the treatment groups was determined as previously described (812,904,1056).

Results

NR4A1 regulates β1-integrin gene expression

Figure 91.

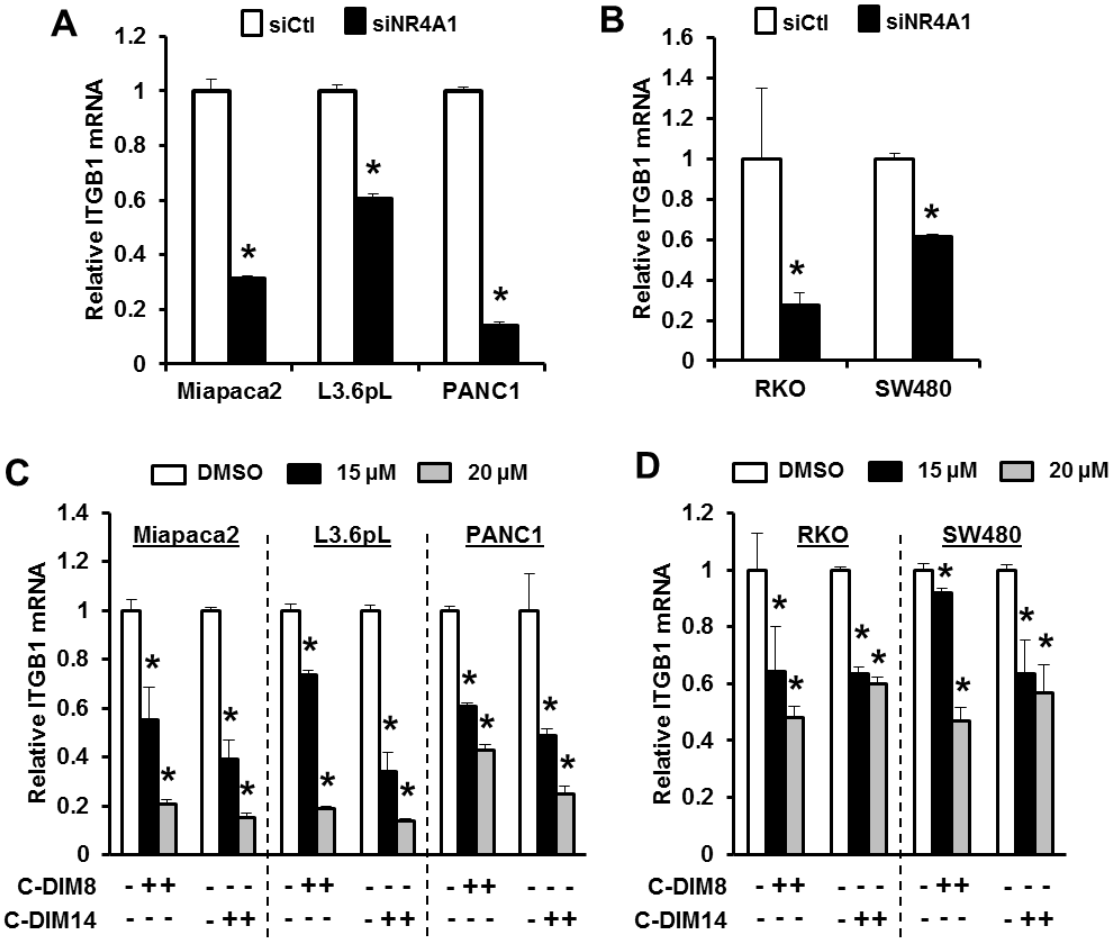

Figure 91. β1-Integrin is an NR4A1-regulated gene. Pancreatic (A) and colon (B) cancer cells were transfected with siNR4A1, and β1-integrin (ITGB1) mRNA levels were determined by real time PCR as outlined in the Materials and Methods. Pancreatic (C) and colon (D) cancer cells were treated with 15 and 20 μM DIM-C-pPhOH (C-DIM8) and DIM-C-pPhCO$_2$Me (C-DIM14) for 24 hr, and mRNA levels were determined by real time PCR as outlined in the Materials and Methods. Results are expressed means ± SE for three determinations per treatment group and significantly ($p<0.05$) decreased expression is indicated (*).

Knockdown of NR4A1 by RNAi in pancreatic (Panc1, L3.6pL and MiaPaCa2) (Fig. 91A) and colon (RKO and SW480) (Fig. 91B) cancer cells

significantly decreased β1-integrin (ITGB1) mRNA levels as determined by real time PCR. Moreover, treatment of the same cell lines with 15 and 20 μM of the two C-DIM/NR4A1 antagonists DIM-C-pPhOH (C-DIM8) and the *p*-carboxymethyl analog (DIM-C-pPhCO_2Me, C-DIM14) also decreased expression of β1-integrin mRNA levels (Figs. 91C and 91D). Western blot analysis of whole cell lysates from Panc1, L3.6pL and MiaPaCa2 cells transfected with siCtl (non-specific oligonucleotide) or siNR4A1 showed that loss of NR4A1 resulted in decreased expression of β1-integrin, phosphorylated FAK (pFAK) and α5-integrin in all three cell lines (Fig. 92A). Moreover, after knockdown of β1-integrin (siβ1-integrin), we also observed decreased expression of β1-integrin, α5-integrin and pFAK (downstream from β1-integrin).

Similar results were observed in RKO and SW480 cells; knockdown of NR4A1 decreased β1-integrin, α5-integrin and pFAK, and knockdown of β1-integrin also decreased pFAK and α5-integrin (Fig. 92B). Treatment of the pancreatic cancer cell lines with 15 or 20 μM DIM-C-pPhCO_2Me and DIM-C-pPhOH also decreased expression of β1-integrin, pFAK and α5-integrin (Figs. 92C and 92D) and the NR4A1 antagonists caused similar effects in RKO and SW480 cells (Figs. 92E and 92F) demonstrating that NR4A1 regulates β1-integrin in both pancreatic and colon cancer cells and that knockdown of NR4A1 (siNR4A1) or treatment with NR4A1 antagonist decreases β1-integrin expression. We also observed that DIM-C-pPhOH decreased β1-integrin and α5-integrin

expression in tumor lysates from an orthotopic pancreatic cancer model using L3.6pL cells (Suppl. Fig. A-10) (804). A previous study reported that knockdown of β1-integrin also decreased α5-integrin (1057).

2NR4A1 antagonists inhibit cancer cell adhesion and migration

Figure 92.

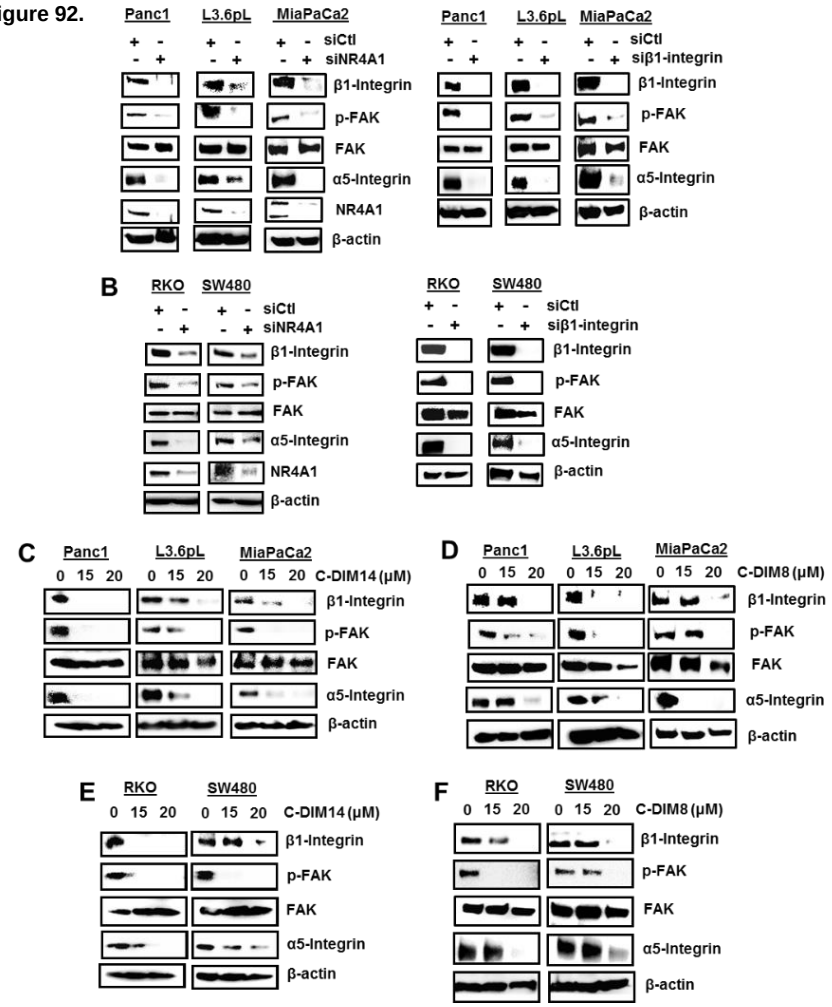

Figure 92. NR4A1 regulates β1-integrin and β1-integrin-regulated genes. Pancreatic (A) and colon (B) cancer cells were transfected with siNR4A1 or siβ1-integrin, and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. Pancreatic cancer cells were treated with DIM-C-pPhCO$_2$Me (C-DIM14) (C) and DIM-C-pPhOH (C-DIM8) (D), and colon cancer cells were treated with the same C-DIMs (E and F) for 24 hr, and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. Similar results were observed in more than one experiment for each panel.

β1-Integrin regulates multiple pathways including cell migration and adhesion, and knockdown of NR4A1 by RNAi significantly decreased migration of Panc1, L3.6pL and MiaPaCa2 cells in a Boyden chamber assay (Fig. 93A) and similar results were observed in RKO and SW480 colon cancer cells transfected with siNR4A1 (Fig. 93B). Migration of Panc1, L3.6pL and MiaPaCa2 cells was also significantly decreased after treatment with the NR4A1 antagonists DIM-C-pPhOH and DIM-C-pPhCO_2Me (Figs. 93C and 93D) and similar results were observed in RKO and SW480 cells treated with the same compounds (Figs. 93E and 93F). Results demonstrate a dose dependent response in migration and this coincided with the data obtained from siNR4A1 treatment. We also used pancreatic cancer cell lines BxPC3 and the colon cancer cell line HCT116 and similar results were obtained (data not shown) with siNR4A1 and using the NR4A1 anatgonists DIM-C-pPhOH and DIM-C-pPhCO_2Me.

Figure 93.

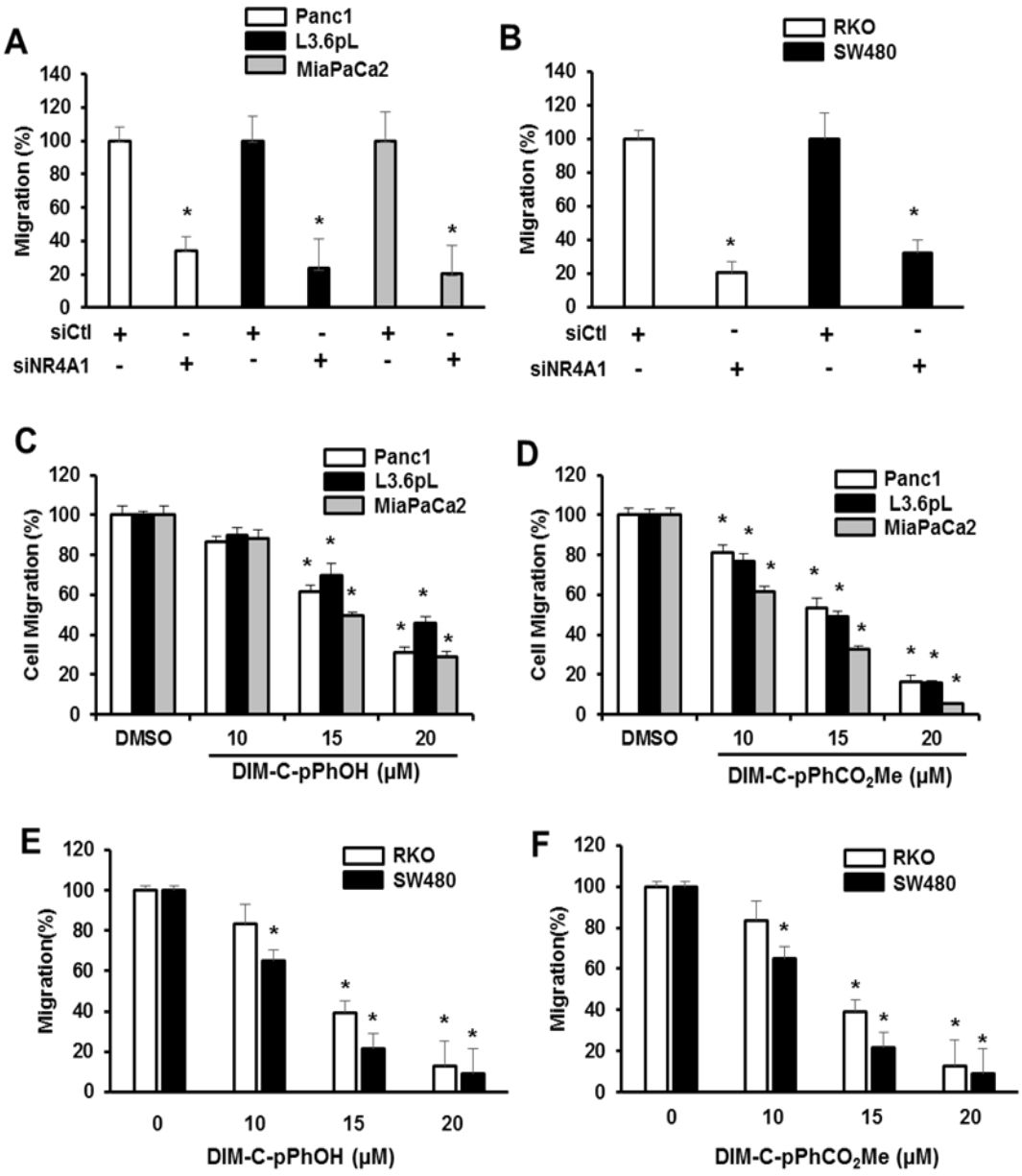

Figure 93. NR4A1 regulates pancreatic and colon cancer cell migration. Pancreatic (A) and colon (B) cancer cells were transfected with siCtl (control) or siNR4A1, and migration was determined in a Boyden chamber assay. Pancreatic cancer cells were treated with DIM-C-pPhOH (C) and DIM-C-pPhCO$_2$Me (D) and colon cancer cells were treated with DIM-C-pPhOH (E) and DIM-C-pPhCO$_2$Me (F) for 24 hr and migration was determined in a Boyden chamber assay as outlined in the Materials and Methods. Each experiment was carried out in triplicate, and results are expressed as mean ± SE and significantly ($p<0.05$) decreases in migration are indicated (*).

We also investigated the antimigratory effects of DIM-C-pPhCO$_2$Me in pancreatic cancer cells after knockdown of NR4A1 or β1-integrin. DIM-C-pPhCO$_2$Me decreased migration; however, in cells depleted of NR4A1 or β1-integrin minimum inhibition was observed in cells after treatment with DIM-C-pPhCO$_2$Me (Fig. 94A) and similar results were observed in colon cancer cells (Fig. 94B) and also with DIM-C-pPhOH (Suppl. Fig. A-11). These results not only confirm the importance of both NR4A1 and β1-integrin in cell migration but also demonstrate that in the absence of β1-integrin, DIM-C-pPhCO$_2$Me-mediated antagonism of other NR4A1-mediated pro-oncogenic pathways has minimal effects on cell migration. Moreover, in pancreatic and colon cancer cells transfected with siNR4A1, the resulting decreased migration was rescued by β1-integrin (ITGB1) gene overexpression (Fig. 94C). Previous studies show that NR4A1-dependent anticancer activities of C-DIMs in colon and pancreatic cancer cells were due to modulation of nuclear NR4A1-mediated changes in gene expression (803-806) and this contrasted to several apoptosis-inducing agents which cause nuclear export of NR4A1 [(reviewed in 905)]. This was also confirmed in this study showing that the inhibitory effects of DIM-C-pPhCO$_2$Me on cell migration in pancreatic (Fig. 94D) and colon (Fig. 94E) cancer cells were comparable in the absence or presence of the nuclear export inhibitor leptomycin B (LMB) demonstrating that the effects of the antagonist on NR4A1 (and β1-integrin) were nuclear effects and not associated with receptor export.

Figure 94.

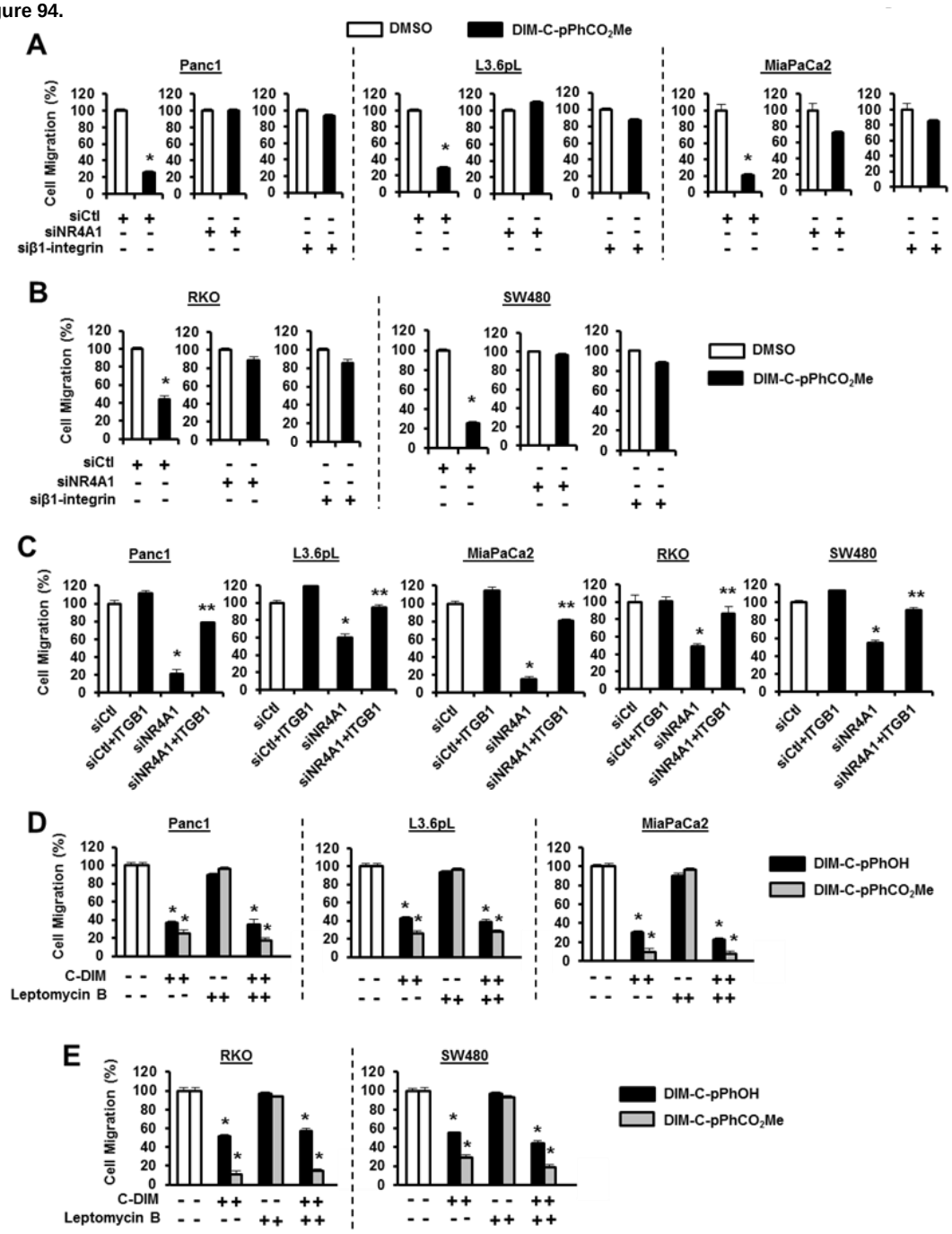

Figure 94. β1-Integrin-dependent migration is dependent on NR4A1. Pancreatic (A) and colon (B) cancer cells were treated with DMSO or 20 μM DIM-C-pPhCO$_2$Me alone or after transfection with siCtl (control), siNR4A1 or siβ1-integrin, and migration was determined in a Boyden chamber assay as outlined in the Materials and Methods. (C) Pancreatic and colon cancer cells were transfected with siCtl (control), siCtl+β1-integrin (ITGB1) expression plasmid, siNR4A1, and siNR4A1+β1-integrin expression plasmid, and cell migration was determined in a Boyden chamber assay. Pancreatic (D) and colon (E) cancer cells were treated with DMSO, 20 μM DIM-C-pPhOH or DIM-C-pPhCO$_2$Me alone or in combination with leptomycin for 24 hr, and migration was determined in a Boyden chamber assay. Results are expressed as mean ± SE for three separate determinations for each treatment group and significant (p<0.05) inhibition of migration (*) and rescue by β1-integrin overexpression (**) (C) are indicated.

Extracellular matrix components such as fibronectin are ligands for integrins and induce α- and β-integrin heterodimer formation and activation of downstream pathways. Fibronectin-induced adhesion was investigated using BD-coated fibronectin in 24-well plates and transfection of pancreatic (Fig. 95A) and colon (Fig. 95B) cancer cells with siNR4A1 or siβ1-integrin significantly decreased adhesion demonstrating that like β1-integrin, NR4A1 plays a role in cell adhesion. This was further confirmed in pancreatic (Fig. 95C) and colon (Fig. 95D) cancer cells treated with DIM-C-pPhOH and DIM-C-pPhCO_2Me which significantly inhibited fibronectin-mediated adhesion Thus, NR4A1 regulation of β1-integrin maintains adhesion in cancer cells and this can also be significantly inhibited by NR4A1 antagonists or knockdown of NR4A1.

Figure 95.

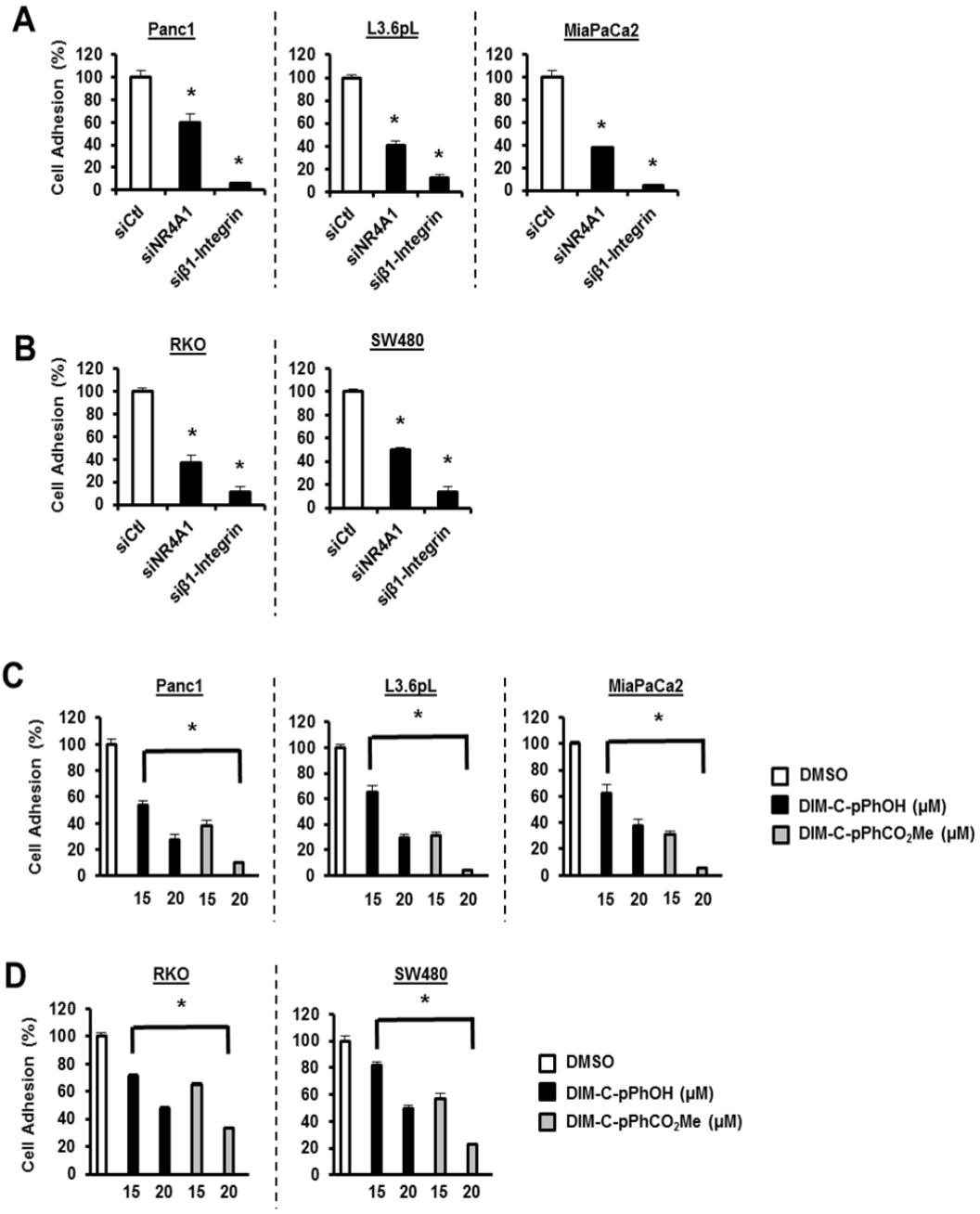

Figure 95. NR4A1 inactivation decreases cancer cell adhesion. Pancreatic (A) and colon (B) cancer cells were transfected with siCtl (control), siNR4A1 and siβ1-integrin, and cell adhesion was determined as outlined in the Materials and Methods. Pancreatic (C) and colon (D) cancer cells were treated with DIM-C-pPhOH or DIM-C-pPhCO$_2$Me for 24 hr, and cell adhesion was determined as outlined in the Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group and significant ($p<0.05$) inhibition of adhesion is indicated (*).

Mechanism of NR4A1 regulation of β1-integrin

Previous studies in breast cancer cells showed that β1-integrin is regulated by a NR4A1/p300/Sp1 complex bound to a proximal GC-rich region of the β1-integrin promoter (Fig. 96A) (903) and this same complex also regulates survivin expression in pancreatic and colon cancer cells (804,806). The functional role of these complexes was investigated by RNAi, and knockdown of p300 decreased β1-integrin protein expression (Fig. 96B) in the five pancreatic and colon cancer cell lines and this paralleled the effects observed after knockdown of NR4A1 or treatment with NR4A1 antagonists (Fig. 92). In a separate experiment, cells were transfected with siSp1, and decreased expression of β1-integrin was observed only in SW480 cells (Fig. 96C) and this corresponded with the role of Sp1 in regulating β1-integrin in breast cancer cells (903). Interestingly, knockdown of Sp1 also decreased expression of p300 in SW480 cells and this may also contribute to the decreased expression of β1-integrin. However, since Sp1 knockdown did not affect β1-integrin expression in Panc1, L3.6pL, MiaPaCa2 and RKO cells (data not shown), we further investigated the role of Sp3 and Sp4 in regulation of β1-integrin using lysates from a previous study on suppression of Sp proteins (763). Figure 6D shows that knockdown of Sp4 and all three Sp proteins combined (siSp1/3/4) decreased β1-integrin expression in all four cell lines and in L3.6pL cells, knockdown of Sp3 also decreased β1-integrin expression. Thus, the role of Sp1, Sp3 and Sp4 in NR4A1-dependent regulation of β1-integrin is cell

context dependent. The effects of Sp1, Sp3 and Sp4 (singly and combined) knockdown in RKO cells were not previously determined and it was evident that Sp3 and Sp4 but not Sp1 coregulated each other (Fig. 96E) from their respective GC-rich promoters (1028,1071). Figure 96F summarizes results of ChIP assays on interactions with the GC-rich sequence of the β1-integrin promoter in all five pancreatic and colon cancer cell lines. In untreated cells, pol II, NR4A1, Sp1, Sp3, Sp4 and p300 were bound to the GC-rich region with one exception; Sp3 binding was barely detectable in SW480 cells.

Treatment with the two NR4A1 antagonists (C-DIM8 and C-DIM14) induced similar effects on promoter interactions but differed in the magnitude of the response for some factors. Treatment with NR4A1 antagonists decreased pol II and NR4A1 interaction to the β1-integrin promoter in all five cell lines and Sp1 was decreased only in SW480 cells. Sp3 interactions were variable (increased, decreased and unchanged) and Sp4 was decreased in all cells with the exception of SW480 cells. The NR4A1 antagonists decreased p300 interactions with the β1-integrin promoter in SW480 and RKO colon cancer cells but not in the pancreatic cancer cell lines. These results demonstrate that the effects of NR4A1 antagonists on the NR4A1/p300/Sp interactions with the β1-integrin promoter are complex and cell context dependent with DNA-bound Sp4 being a major factor based on the knockdown and ChIP assays (Fig. 96D). This is the first example of an important role for NR4A1/Sp4-mediated transactivation, and examples of other genes regulated by this complex are currently being investigated.

Figure 96.

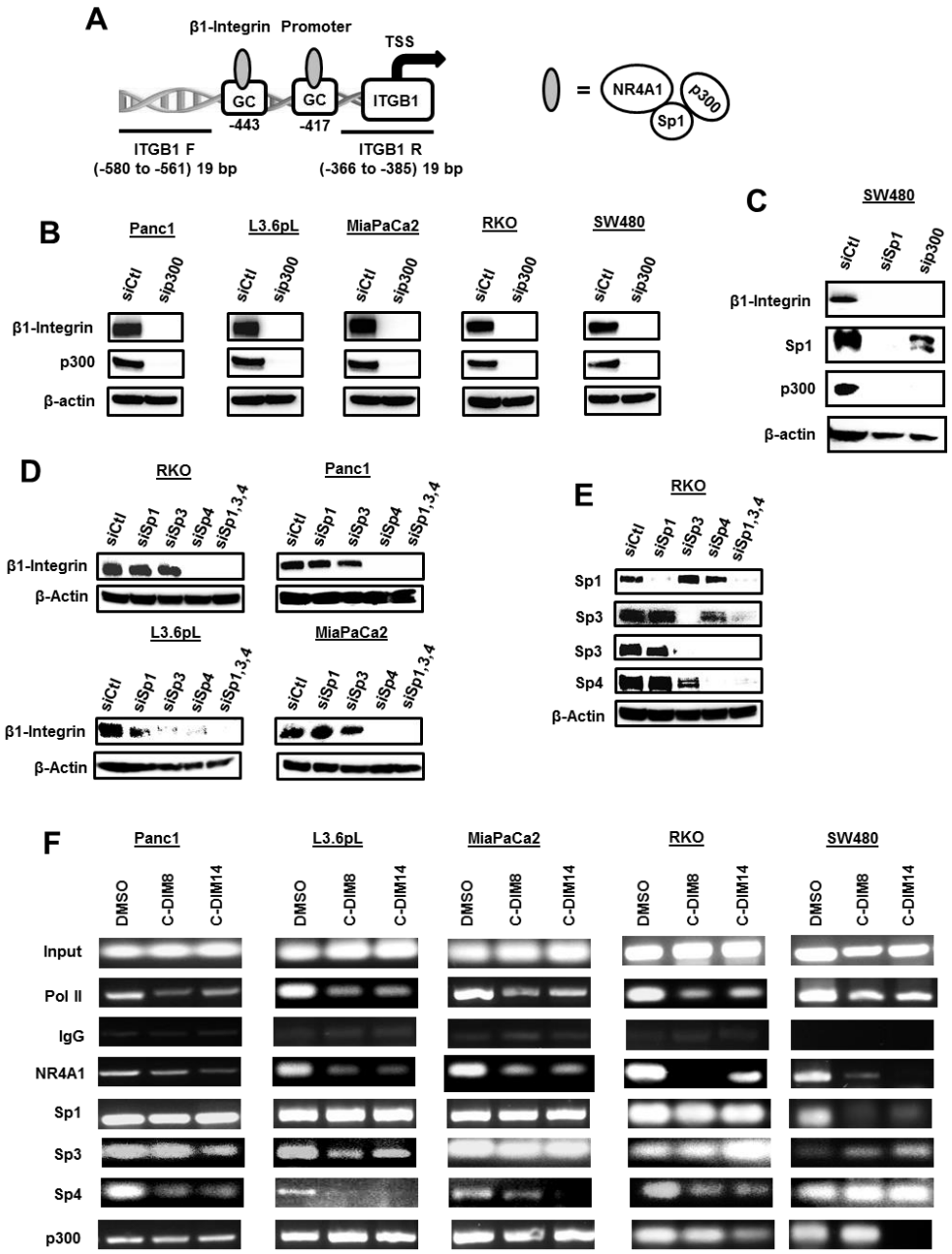

Figure 96. Mechanism of NR4A1 regulation of β1-integrin gene expression. (A) β1-Integrin promoter and proximal GC-rich sequence. (B) Pancreatic and colon cancer cells were transfected with siCtl (control) and sip300, and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (C) SW480 cells were transfected with siCtl (control), siSp1 and sip300, and whole cell lysates were analyzed by western blots. (D) RKO, Panc1, L3.6pL and MiaPaCa2 lysates from cells transfected with siCtl (control), siSp1, siSp3, siSp4 or siSp1,3,4 were obtained from a previous study (42) and analyzed by western blots. (E) Using a similar protocol, Sp proteins were also knockdowned in RKO cells and analyzed for different Sp proteins by western blots. [Note: RKO cells were not included in a previous study on the effects of Sp knockdown in multiple cancer cell lines (42).] (F) Pancreatic and colon cancer cells were analyzed in ChIP assays to determine interactions with the GC-rich region of the β1-integrin promoter as outlined in the Materials and Methods. C-DIM8 = DIM-C-pPhOH; C-DIM14 = DIM-C-pPhCO$_2$Me.

Discussion

Integrins are heterodimeric cell surface receptors that consist of an α- and β-subunit and these receptors play critical roles in maintaining cellular homeostasis and also in several human pathologies. There are 18 different α and 8 different β integrin subunits and among the 24 α/β-integrin heterodimers, β1-integrin forms the largest subgroup of 12 receptor complexes (840-842,844,1072). β1-Integrin heterodimers interact with other signaling pathways and activate multiple genes and kinases such as focal adhesion kinase resulting in the induction of cell adhesion, migration and invasion. The role of integrins in multiple diseases has spurred studies on development of drugs that target integrins and these include antibodies, peptides that inhibit heterodimer formation, and other small molecules (841,842,844). However, the success of these agents in cancer chemotherapy has been limited and integrin inhibitors have not been approved for clinical applications in cancer chemotherapy.

C-DIMs have been identified as a novel class of NR4A1 ligands that modulate nuclear NR4A1-mediated gene expression and associated pathways (803,804,806,812, 904,1052,903). DIM-C-pPhOH and DIM-C-pPhCO$_2$Me inhibit mTOR, decrease cell proliferation and survival, and induce ROS in multiple cancer cell lines where these compounds act as NR4A1 antagonists (803,805). Our recent study showed that these NR4A1 ligands also inhibit breast cancer cell migration through inhibition of β1-integrin expression which is an NR4A1-

regulated gene in breast cancer cells (903). There is also evidence that β1-integrin is a negative prognostic factor for colon and pancreatic patients and plays an important role in cancer cell migration/invasion and metastasis (1056-1067). It was evident from the results of RNAi studies that decreased expression of NR4A1 resulted in decreased expression of β1-integrin RNA and protein, α5-integrin and a β1-integrin-activated FAK gene phosphorylation (Figs. 91A, 91B, 92A and 92E). Moreover, siNR4A1 also decreased β1-integrin-regulated migration and adhesion (Figs. 93 and 95) and the effects of NR4A1 or β1-integrin knockdown were comparable indicating that NR4A1 regulates β1-integrin in pancreatic and colon cancer cells as previously observed for breast cancer cell lines (903). We also observed that knockdown of NR4A1 by RNAi or treatment with DIM-C-pPhOH and DIM-C-pPhCO$_2$Me gave comparable results demonstrating that these C-DIMs act as NR4A1 antagonists to decrease β1-integrin expression and thereby represent a novel class of agents targeting β1-integrin in pancreatic and colon cancer.

Although NR4A1 regulates expression of genes through binding as a monomer or dimer to cognate response elements in breast cancer cells, NR4A1 regulates expression of β1-integrin by cooperative interactions of NR4A1 and p300 with promoter DNA (GC-rich)-bound Sp1 (903). NR4A1/p300 also induced survivin expression through interactions with Sp1 bound to the GC-rich survivin promoter (804). Results of RNAi and ChIP assays showed that in SW480 colon cancer cells, β1-integrin was regulated by an NR4A1/p300/Sp1 complex bound to

the proximal GC-rich site of the β1-integrin promoter (Fig. 96A). ChIP assays showed that Sp1, Sp3 and Sp4 bound the proximal GC-rich site in the β1-integrin promoter; however, it is clear from Sp knockdown studies that the functional Sp protein was cell context dependent (Fig. 96C and 96D). Thus, Sp4 and to a lesser extent Sp3 in combination with NR4A1 and p300 also regulate β1-integrin expression is some colon and pancreatic cancer cell lines and this contrasts to previous studies in breast cancer cells where β1-integrin is an NR4A1/p300/Sp-1 regulated gene (903). The reason for the cell selective and functional role of Sp1, Sp3 and Sp4 in regulating β1-integrin expression via an NR4A1/p300/Sp complex is unclear and is currently being investigated.

In summary, results of this study demonstrate the role of NR4A1 in regulation of pancreatic and colon cancer cell migration through transcriptional activation of β1-integrin which is an NR4A1/p300/Sp-regulated gene. This observation is consistent with previous studies demonstrating that NR4A1 regulates multiple pro-oncogenic genes/pathways in solid tumors. We also show that C-DIM/NR4A1 antagonists represent a novel class of drugs that target β1-integrin in pancreatic and colon cancer, and current studies are focused on developing these compounds for future clinical applications.

CHAPTER VIII

PENFLURIDOL REPRESSES INTEGRIN EXPRESSION IN BREAST CANCER THROUGH INDUCTION OF REACTIVE OXYGEN SPECIES AND DOWNREGULATION OF SP TRANSCRIPTION FACTORS

Introduction

Repositioning clinically-approved drugs for cancer chemotherapy has several advantages including a more rapid drug approval process coupled with potential development of mechanism-based compounds that can be clinically used to target important pro-oncogenic pathways. This approach has been particularly successful with non-steroidal antiinflammatory drugs (NSAIDs) and antidiabetics such as metformin (1073,1074). Phenothiazine-derived antipsychotic drugs such as thioridazine and chlorpromazine exhibit anticarcinogenic activity in several different cancer cell lines (1075-1081). More recent studies have demonstrated that penfluridol, another typical antipsychotic drug of the diphenylbutylpiperidine class, also inhibits breast and pancreatic cancer cell growth (1081,1082). For example, in pancreatic cancer a series of phenothiazene analogs induced apoptosis and inhibited growth and colon formation, and more detailed studies with penfluridol indicated that induction of protein phosphatase 2A (PP2A) was a key effect of this compound (1081). Penfluridol exhibited antimetastatic activity in triple negative breast cancer cells

and inhibited tumor growth and brain metastasis in three different *in vivo* models and the key elements were inhibition of α6- and β4-integrin expression (1082). However, the mechanisms of the penfluridol-induced responses were not well defined, and this limits potential clinical applications of the compound.

Recent studies in this laboratory showed that β1- and β3-integrin expression in breast cancer cells is regulated by specificity protein 1 (Sp1) transcription factor (TF) in combination with the orphan nuclear receptor 4A1 (NR4A1, Nur77, TR3) which acts as a nuclear cofactor (903). Many of the effects observed in breast and other cancer cell lines treated with penfluridol and other phenothiazine derivatives are similar to that observed after knockdown of Sp transcription factors Sp1, Sp3 or Sp4 or after treatment with agents that target Sp TFs (645,646,648,763,996,903,1082-1084). For example, knockdown of Sp1, Sp3 or Sp4 individually or combined decreased proliferation and migration/invasion of breast (MDA-MB-231 and SKBR3) and other cancer cell lines (763) and similar results were observed for drugs that repress Sp TF expression (645,646,648,996,1083,1084). Moreover, the effects of penfluridol and other phenothazines on inhibition of several genes including cyclin D1, bcl-2, vascular endothelial growth factor (VEGF) receptors, myc and activation/cleavage caspase-3/PARP (1075-1082) have also been observed after Sp knockdown (645,646,648,763,996,903,1082-1084). It was recently reported that the antimetastatic activity of penfluridol in triple negative breast cancer cells was related to downregulation of α6- and β4-integrin expression (1082); however,

since both integrin gene promoters are GC-rich, it is possible that Sp1 and other Sp TFs may regulate expression of α6- and β4-integrins as well as α5-integrin (1085-1087).

Therefore, we hypothesize that the mechanism of action of penfluridol as an antimetastatic agent for triple negative breast cancer is due to downregulation of Sp TFs. This hypothesis was confirmed in this study which shows that penfluridol induces reactive oxygen species (ROS) in breast cancer cells and ROS-dependent downregulation of Sp1, Sp3 and Sp4 and Sp-dependent genes including α6-, α5-, β1- and β4-integrins which are also coregulated by NR4A1.

Materials and Methods

Cell lines and antibodies

Breast cancer (SKBR3, MDA-MB-231) cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained 37□C in the presence of 5% CO2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic. Dulbecco's Modified Eagle's Medium was purchased from GenDepot (Barker, TX). Penfluridol and 36% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Glutathione (GSH) reduced free acid was purchased from Millipore (Temecula, CA). Hematoxylin was purchased from Vector Laboratories (Burlingame, CA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from

Biotium (Hayward, CA). Antibodies were purchased as outlined in Supplementary Table 1.

Cell proliferation assay and Annexin V staining

Cell proliferation assays were carried out as described previously (11-13), and changes in cell number were determined by Coulter Z1 cell counter. Annexin V staining used the Vybrant apoptosis kit according to the manufacturer's protocol.

Boyden chamber assay

SKBR3 and MDA-MB-231 cancer cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hr. Cells were seeded and subsequently treated with varying concentrations of penfluridol for 24 hr (+/- GSH, 3 hr prior to treatment). Cells were trypsinized, counted, placed in 12-well 8.0 µm pore ThinCerts from Greiner Bio-one (Monroe , NC), allowed to migrate for 24 hr, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted as described.

RT-PCR

miRNA was isolated using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. Quantification of miRNA

(RNU6B and miR-17, miR-20a, and miR-27a) was done using the TaqMan miRNA assay kit (Life Technologies) according to the manufacturer's protocol with real-time PCR. U6 small nuclear RNA was used as a control to determine relative miRNA expression.

Western blot analysis

SKBR3 and MDA-MB-231 cancer cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hr. Cells were treated with varying doses of penfluridol for 24 hr. Cells were analyzed by western blot as described previously.

Small interfering RNA interference assay

siRNAs used are outlined in Supplementary Table 4. SiRNA experiments were conducted as described previously.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. SKBR3 and MDA-MB-231 cells were treated with DMSO, DIM-C-pPhOH (15 or 20 μM), DIM-C-pPhCO2Me (15 or 20 □M) for 24 hr, or penfluridol (5 μM) for 3 or 6 hr. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by

addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (~200 to 1,500 bp). The sonicated chromatin was immunoprecipitated with normal IgG, p300, Sp1, Sp3, Sp4, NR4A1, or RNA polymerase II antibodies and protein A-conjugated magnetic beads at 4°C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. DNA was prepared by proteinase K digestion followed by PCR amplification. Primers used for detecting PCR products are listed in Supplementary Table 1. PCR products were resolved on a 2% agarose gel in the presence of RGB-4103 GelRed Nucleic Acid Stain.

Xenograft studies

Female athymic nude mice 4-6 old were purchased, and MDA-MB-231 cancer cells (2.0 x106 cells) were suspended in Matrigel (1:1 ratio) and injected into the mammary fat pad of athymic nude mice. When tumors became palpable (150-200 mm3), mice were randomly assigned to control (corn oil vehicle) and penfluridol (10 mg/kg/day), and then treated every day for 19 days. Tumor volumes, tumor weights, and tumor lysates were determined and analyzed as previously described (645,646,648).

Generation and measurement of ROS

Cellular ROS levels were ascertained using the cell permeable probe CM-H2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen (Grand Island, NY). CM-H2DCFDA is non-fluorescent until cleavage of the acetyl groups by intracellular esterases and oxidation that transpires within the cell. Cells were pretreated for 3 hr with antioxidant glutathione (GSH, 5 mM) or N-acetyl cysteine (NAC, 5 mM), then subsequently treated with respective doses of penfluridol for 3, 6 or 24 hr. The antioxidant at the time of treatment of penfluridol was not washed away and penfluridol treatment was with antioxidant cotreatment. Following treatment, cells plated on a 6-well culture plate were trypsinized, neutralized, then loaded with 10 µM of probe for 20 min, washed once with serum free medium, and then ROS was measured by flow cytometry using Accuri's C6 Flow Cytometer (Accuri, Ann Arbor, MI).

Plasmids

pCDH-puro-cMyc was a gift from Jialiang Wang (Addgene plasmid #46970) and pCDH-CMV-MCS-EF1-Puro was obtained from System Biosciences (Palo Alto, CA). MDA-MB-231 and SKBR3 breast cancer cells were seeded (1.2 x 105 per well) in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% charcoal-stripped fetal bovine serum and left to attach for 24 hr. Plasmid transfection was performed using Lipofectamine 2000 reagent

according to the manufacturer's protocol. Cells were transfected with c-Myc-overexpressing (pCDH-puro-cMyc) or empty control vector (pCDH-CMV-MCS-EF1-Puro) for 6 hr in fresh 2.5% charcoal-stripped fetal bovine serum DMEM. After 6 hr, medium was then replaced with fresh 2.5% charcoal-stripped fetal bovine serum DMEM and treated with either DMSO or 5 μM penfluridol. Cells were then harvested for western blot analysis.

Statistical analysis

Statistical significance of differences between the treatment groups was determined as previously described (645,646,648).

Results

Drugs that downregulate Sp TFs act through ROS-dependent and -independent pathways (996), and results in Figure 97A show that different concentrations of penfluridol induced ROS in MDA-MB-231 and SKBR3 cells after treatment for 3 and 6 hr. Moreover, after cotreatment with 5 mM GSH, the penfluridol-mediated induction of ROS as detected using the fluorescent probe CM-H_2DCFD4 was significantly attenuated. The role of penfluridol-induced ROS on cell proliferation (Fig. 97B), Annexin V staining (apoptosis) (Fig. 97C), and migration in a Boyden chamber assay (Fig. 97D) was also determined in MDA-MBA-231 and SKBR3 cells treated with penfluridol alone and in combination with GSH. Penfluridol inhibited cell growth and induced Annexin V staining, and these

results were consistent with previous studies. However, we now show that all of these responses were ROS-dependent and significantly attenuated after cotreatment with GSH as well as N-acetyl cysteine (NAC) (Suppl. Fig. A-12).

Figure 97.

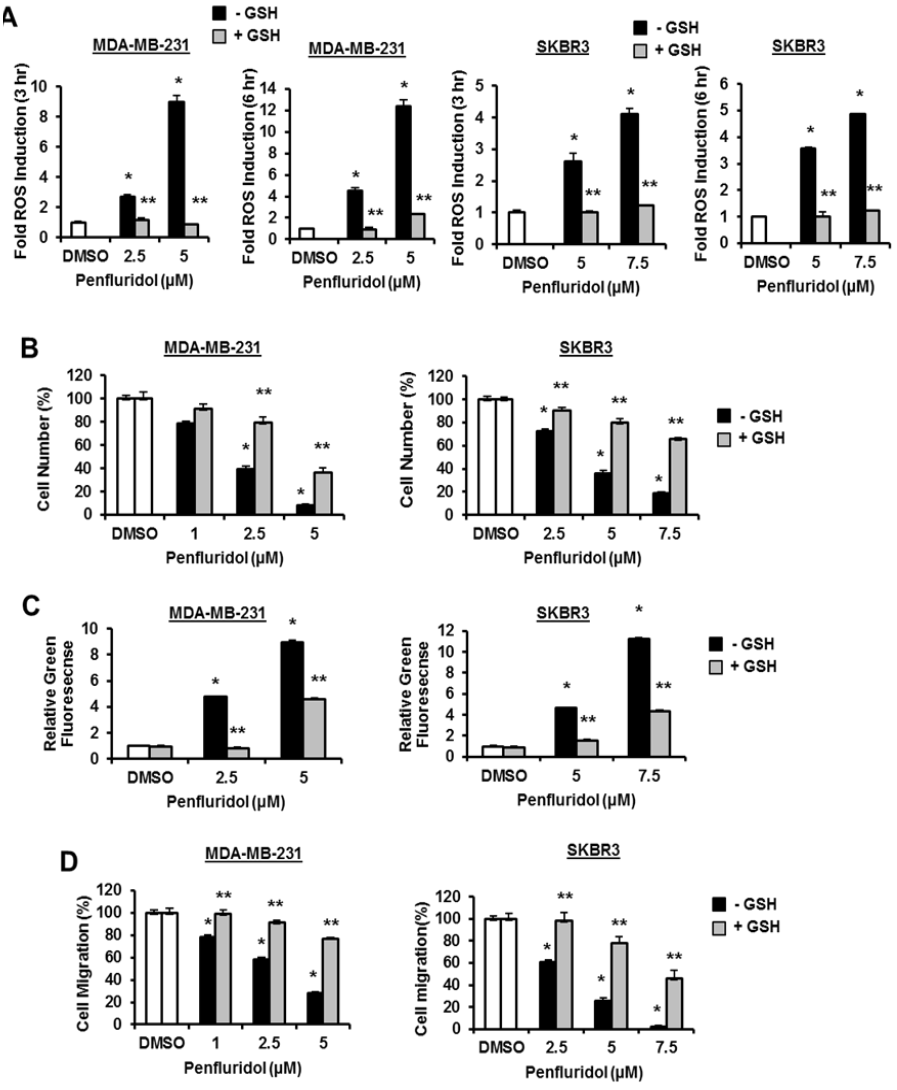

Figure 97. Penfluridol induces ROS and ROS-dependent responses in breast cancer cells. (A) MDA-MB-231 and SKBR3 cells were treated with DMSO, 2.5 or 5.0 μM penfluridol alone, or in combination with 5 mM GSH for 3 hr, and ROS activity was determined as outlined in the Materials and Methods. The cells were treated as outlined above for 24 hr and effects on cell proliferation (B), Annexin V staining (C), and migration (D) in a Boyden chamber assay were determined as outlined in the Materials and Methods. Results are expressed as means ± SE for 3 replicates for each data points and significant (p<0.05) modulation by penfluridol (*) and reversal by GSH (**) are indicated.

Previous studies with multiple ROS-inducing anticancer agents show that ROS decreases Sp TFs (645,646,648,1083,1084), and results in Figures 98A and 98B show that penfluridol decreased expression of Sp1, Sp3 and Sp4 in MDA-MB-231 and SKBR3 cells and cotreatment with GSH attenuated these effects. Similar results were observed with knockdown of c-Myc by siRNA using more than one oligonucleotide (Supplemental Figure A-12) A recent report showed that penfluridol decreased focal adhesion kinase (FAK) phosphorylation and activated (cleaved) caspase 3 in breast cancer cells (1082) and this was also observed in MDA-MB-231 (Fig. 98C) and SKBR3 (Fig. 98D) cells, and these responses were attenuated by cotreatment with GSH. Overexpression of Myc (Suppl. Fig. A-13) or knockdown of ZBTB10 (Suppl. Fig. A-14) also ameliorates Sp protein expression. We also observed that penfluridol induced ROS-dependent PARP cleavage (apoptosis) in both cell lines, confirming that induction of apoptosis and the growth and migration inhibitory effects of penfluridol in breast cancer cells were ROS-dependent. We also demonstrate that H_2O_2 scavenger catalase ameliorates Sp protein expression (Fig. 98E) and the specificity of penfluridol for Sp proteins by inhiting luciferase expression controlled by a promoter, which contains GC rich Sp consensus binding sites (Fig.98F)

Penfluridol activates the ROS ⊣ Myc ⊣ miR-27a/miR-20/17a → ZBTB pathway.

ROS induces a cascade of events which lead to ZBTB10/ZBTB4/ZBTB34-mediated repression of Sp TFs (Fig. 99A) (648,1083), and results in Figure 3B show that penfluridol decreased cMyc expression in MDA-MB-231 and SKBR3 cells and this response was totally reversed after cotreatment with GSH.

Figure 98.

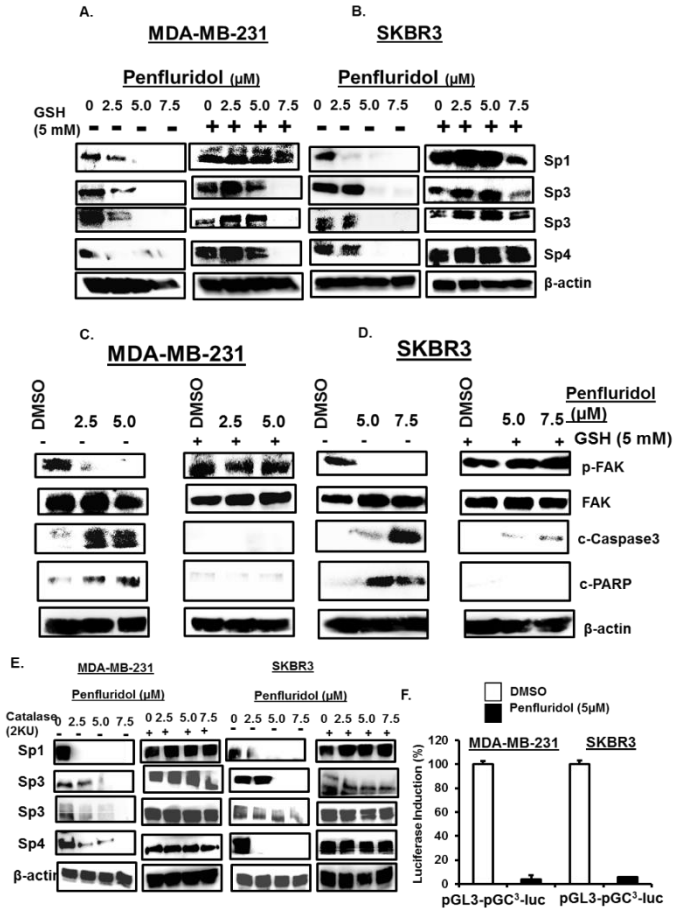

Figure 98. Penfluridol induces ROS-dependent modulation of Sp1, Sp3, Sp4 and other responses in breast cancer cells. MDA-MB-231 and SKBR3 cells were treated with different concentrations of penfluridol alone or in combination with GSH for 24 hr, and whole cell lysates were analyzed for Sp1, Sp3 and Sp4 (A and B, respectively) and other responses (C and D, respectively) as outlined in the Materials and Methods. Pretreatment with catalase also ameliorated penfluridol-mediated Sp downregulation (E). Penfluridol repressed luciferase induction from GC-rich Sp1-consensus promoters (F).

It was reported that ROS-dependent cMyc downregulation is due to rapid shifts of chromatin-modifying complexes from non-GC-rich to GC-rich (e.g. cMyc) gene promoters (648,781,1083), and Figure 99C shows that cMyc was rapidly decreased in MDA-MB-231 and SKBR3 cells after treatment with penfluridol.

Sp1, Sp3 and Sp4 were also rapidly decreased in one or more cell lines and this has previously been observed for other ROS inducers (648,1083) and is consistent with their GC-rich promoters (1027-1029). ChIP analysis showed that pol II association with the GC-rich cMyc promoter was decreased after treatment of MDA-MB-231 and SKBR3 cells with penfluridol for 3 and 6 hr, and this was accompanied by an increase in the inhibitory H3K27me3 and a decrease in the activating H3K4me4 and H4K16Ac histone marks (Fig. 99D). The ROS-dependent decrease in cMyc was accompanied by a decrease in the cMyc-regulated miR-27a and miR-20a/miR-17 after treatment penfluridol and this decrease was also inhibited after cotreatment with GSH (Fig. 99E). Penfluridol-mediated repression of miR-27a and miR-20/miR-17 (part of the miR-17-92 cluster) resulted in induction of the miR-regulated Sp repressors ZBTB10 and ZBTB4, respectively, and this induction response was also attenuated in cells cotreated with GSH. These results demonstrate that the mechanism of penfluridol-induced ROS is initiated by ROS-mediated epigenetic repression of cMyc (Fig. 99A) and this has previously been observed for phenethylisothiocyanate (PEITC) and HDAC inhibitors in pancreatic cancer and rhabdomyosarcoma (RMS) cells, respectively (648,1083).

Figure 99.

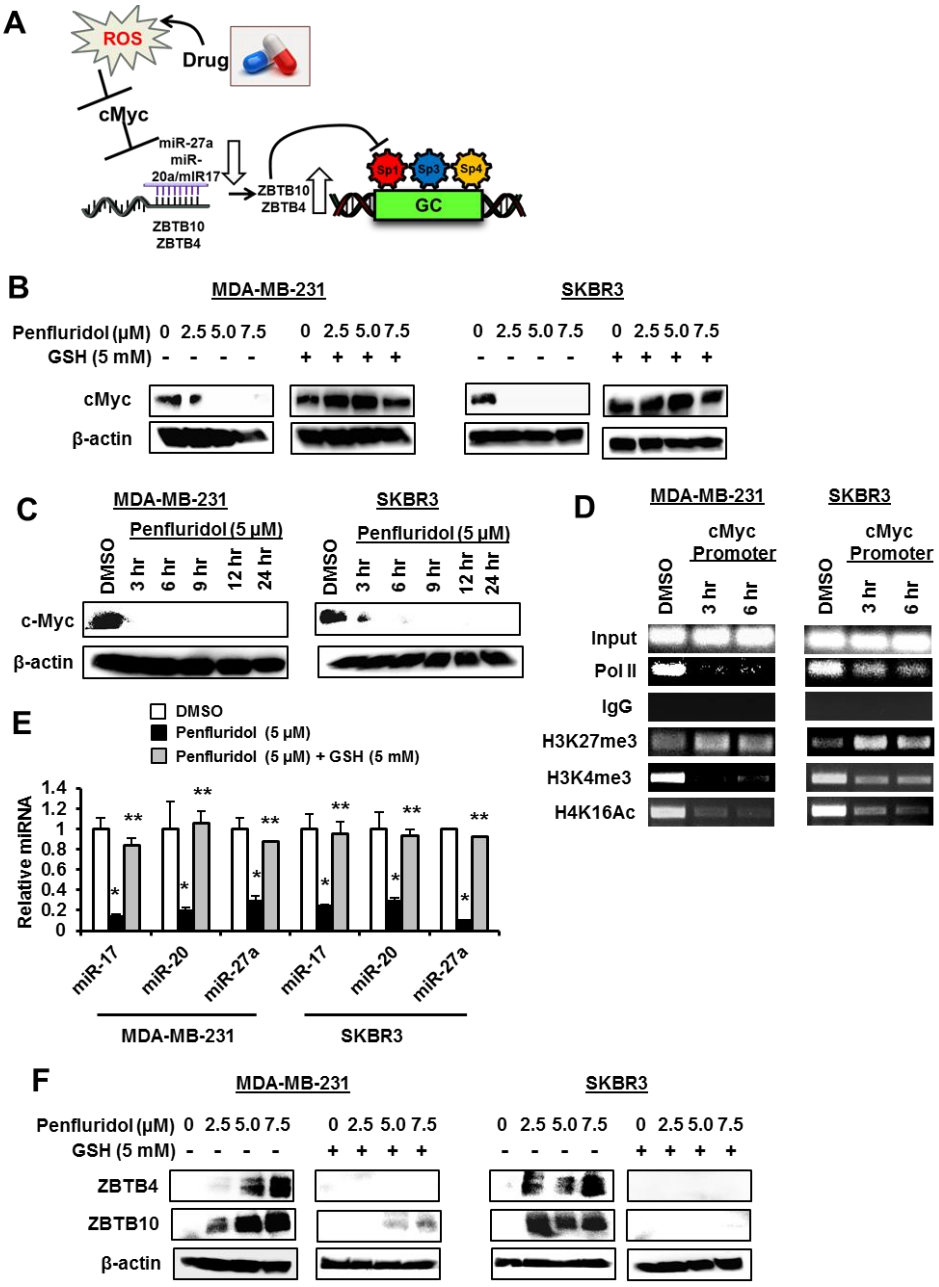

Figure 99. Mechanism of penfluridol-induced Sp downregulation. (A) Molecular mechanism of drug-induced ROS and ROS effects on miR-ZBTB interactions and Sp downregulation (14). MDA-MB-231 and SKBR3 cells were treated with penfluridol alone or in combination with GSH for 24 hr (B) or penfluridol alone for different times (C), and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (D) Cells were treated with 5 μM penfluridol, and effects on interaction with the proximal GC-rich cMyc promoter were by ChIP assays as outlined in the Materials and Methods. (E) Cells were treated as outlined in (B) and expression of microRNAs (miRs) was determined by real time PCR. Penfluridol significantly ($p<0.05$) decreased all miRs and GSH significantly reversed these responses. (F) Cells were treated as outlined in (B) and expression of ZBTB10 and ZBTB4 were determined by western blot analysis as outlined in the Materials and Methods.

Penfluridol induces ROS-dependent repression of integrins

Penfluridol decreases α6- and β4-integrin in breast cancer cells (1082), and our results also show this same response in MDA-MB-231 and SKBR3 cells and cotreatment with GSH attenuated this effect (Fig. 100A). We also observed that α5- and β1-integrin expression was also decreased by penfluridol and rescued after cotreatment with GSH (Figs. 100A and 100B) and this was consistent with our recent studies showing that β1-integrin is an Sp-regulated gene and α5-integrin appears to be coregulated with β1-integrin (903). Confirmation that α6, α5-, β4- and β1-integrin are Sp-regulated genes was determined by RNA interference where knockdown of one or more of Sp1 (siSp1), Sp3 (siSp3), Sp4 (siSp4) or all three Sps (siSp1,3,4) decreased expression of the integrins in MDA-MB-231 (Fig. 100C) and SKBR3 (Fig. 100D) cells. Most of the four integrins were coregulated by two or all three of the Sp TFs; however, α6-integrin and β1-integrin were regulated only by Sp4 (MDA-MB-231) and Sp1 (SKBR3), respectively. In addition, we also show that cMyc indirectly regulated integrin expression through Sp1. Knockdown of cMyc by RNA decreased Sp1, Sp3 and Sp4 expression in MDA-MB-231 and SKBR3 cells (Fig. 100E), and the same treatment also decreased expression of α6-, α5-, β1- and β4-integrin in the same cell lines (Fig. 100F). Thus, penfluridol activation of ROS results in cMyc downregulation, leading to decreased expression of Sp1, Sp3, Sp4 and Sp-regulated genes α6-, α5-, β1- and β4-integrin.

Figure 100.

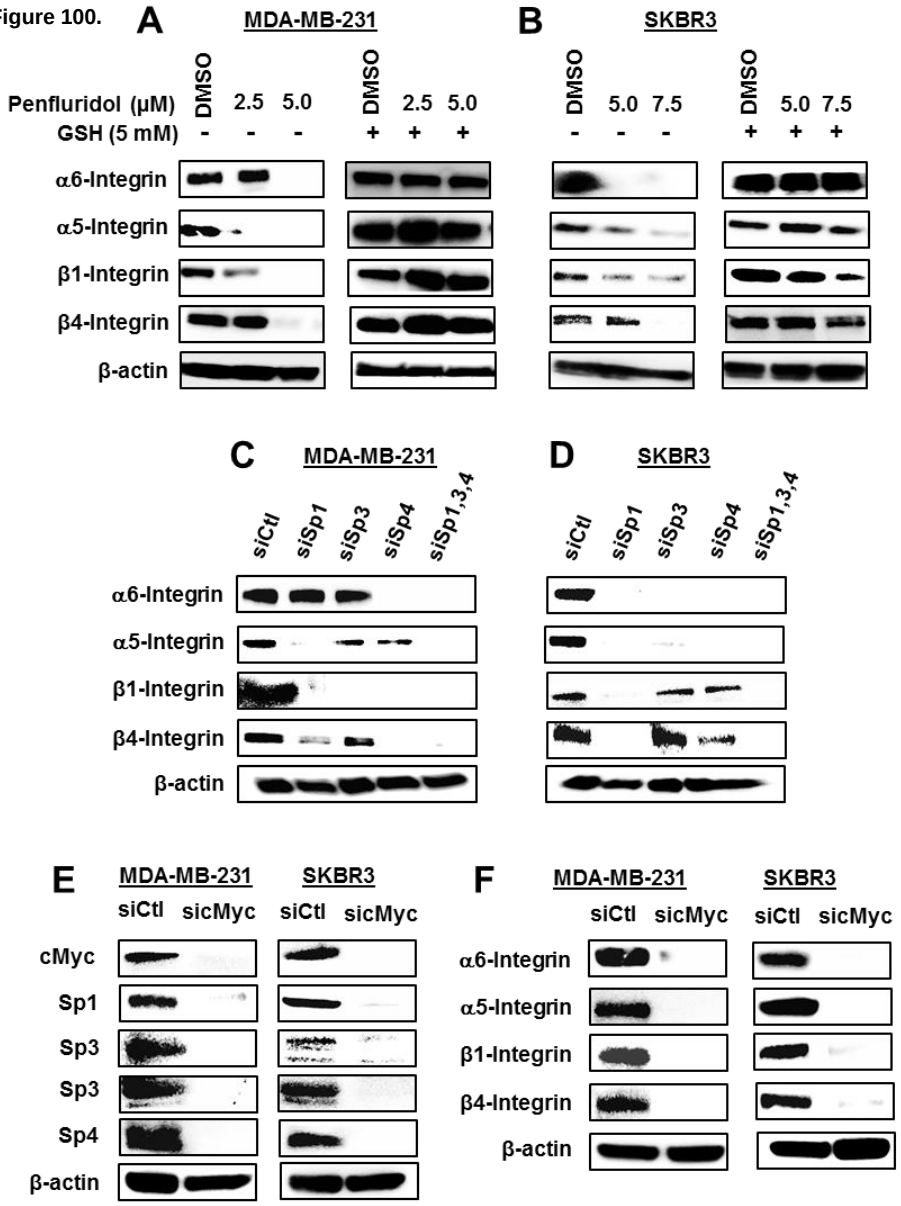

Figure 100. Penfluridol targets integrin expression via an ROS-cMyc (downregulation) pathway. MDA-MB-231 (A) and SKBR3 (B) cells were treated with penfluridol alone or in combination with GSH, and whole cell lysates were analyzed by western blots. Cells were transfected with oligonucleotides specifically targeted to Sp transcription factors [MDA-MB-231(C) and SKBR3 (D)] or cMyc (E and F), and whole cell lysates were analyzed by western blots. Whole cell lysates obtained after Sp knockdown in MDA-MB-231 and SKBR3 cells were generated in a previous study which also reports effects of these oligonucleotides on Sp1, Sp3 and Sp4 expression (C and D) (12).

Since the α6-, α5-, β1- and β4-integrins are Sp-regulated genes, we also investigated the effects of penfluridol on association of pol II and Sp1, Sp3, Sp4 with the GC-rich regions of the integrin gene promoters (Fig. 101A) using a ChIP assay. In addition, since we previously observed that NR4A1 and p300 act as cofactors for Sp-dependent activation of β1-integrin (903), the effects of penfluridol on their association with the integrin promoters was also determined. Figures 101B-101E show that pol II, Sp1, Sp3, Sp4, p300 and NR4A1 were all associated with the α6-, β4-, α5- and β1-integrin gene promoters as determined in a ChIP assay. With the exception of p300, treatment of MDA-MB-231 and SKBR3 cells with penfluridol decreased association of Sp1, Sp3, Sp4 and NR4A1 with the four integrin promoters, whereas the loss of p300 was promoter- and cell context-dependent.

Figure 101.

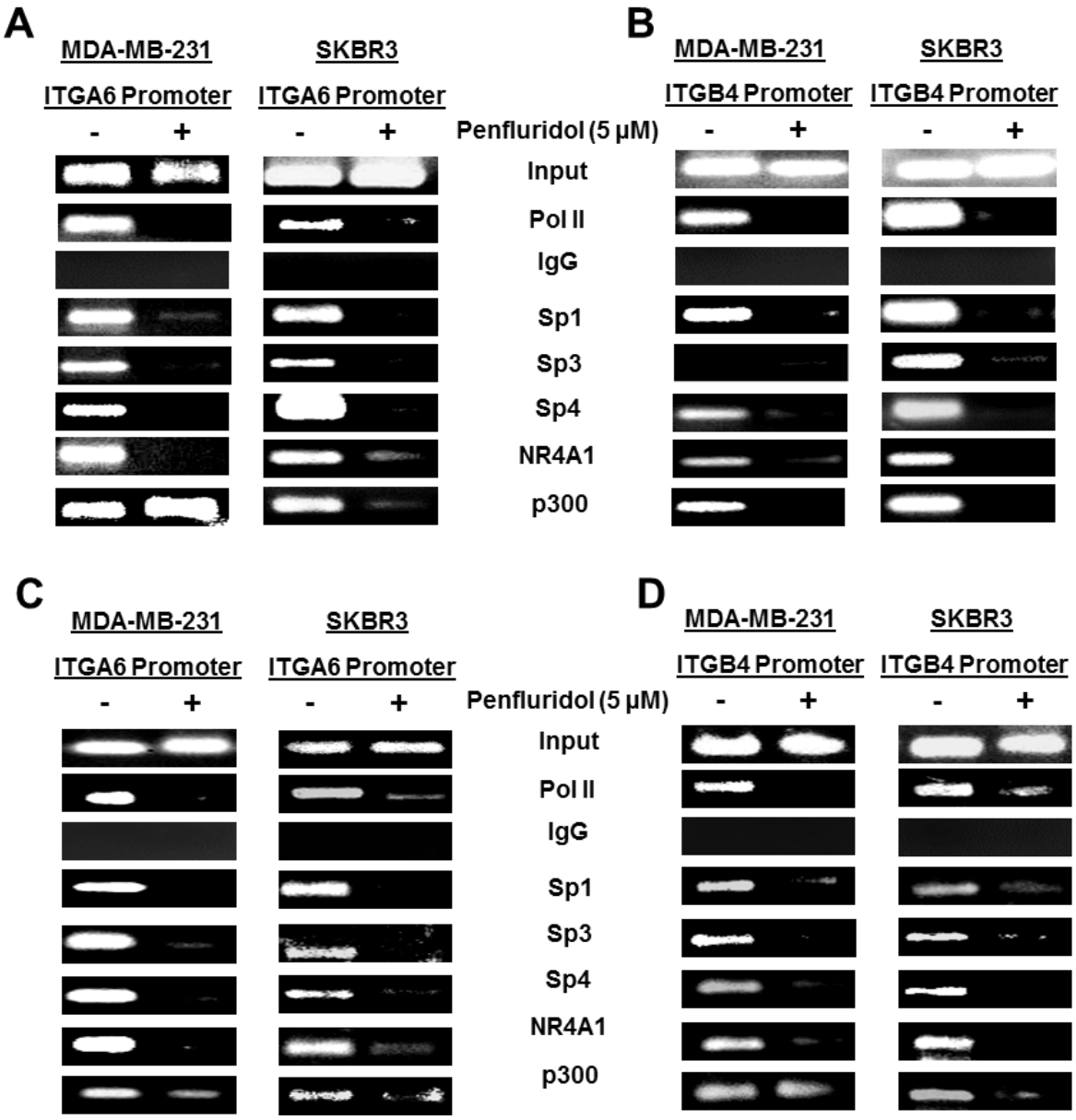

Figure 101. Penfluridol decreases interactions of Sp proteins and other nuclear factors with integrin gene promoters. (A) Outline of GC-rich regions for α6-integrin (ITGA6), β4-integrin (ITGB4), α5-integrin (ITGA5) and β1-integrin (ITGB1) gene promoters and primers targeting these regions. Breast cancer cells were treated with DMSO and 5 μM penfluridol, and interactions of Sp1, Sp3, Sp4 and other nuclear cofactors with the IGFA6 (B), ITGB4 (C), ITGA5 (D) and ITGB1 (E) gene promoters were determined in a ChIP assay as outlined in the Materials and Methods.

NR4A1/Sp regulation of integrins

Previous studies showed that NR4A1/Sp regulate expression of β1- and α5-integrin in breast cancer cells, and knockdown of Sp or NR4A1 or treatment with an NR4A1 antagonist inhibits their expression (903). We also demonstrate that penfluridol does not antagonize NR4A1 as demonstrated by luciferase assay (Suppl. Fig. A-15). Panc1 cells transfected with a GAL4-NR4A1 chimera and a reporter gene containing a GAL4 response element can be used for screening NR4A1 antagonists (804,912), and penfluridol did not inhibit or activate this system (Suppl. Fig. S1). Knockdown of NR4A1 (siNR4A1) or treatment with the NR4A1 antagonists 1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH) or 1,1-bis(3'-indolyl)-1-1(*p*-carbomethoxyphenyl)methane (DIM-C-pPhCO$_2$Me) decreased expression of α6- and β4-integrin in MDA-MB-231 and SKBR3 cells (Fig. 102A and 102B) and similar results were previously observed for β1- and α5-integrin (903). Treatment of MDA-MB-231 and SKBR3 cells with DIM-C-pPhOH (C-DIM8) or DIM-C-pPhCO$_2$Me (C-DIM14) showed that these compounds induced loss of pol II from the α6- (Fig. 102C), β4- (Fig. 102D) and α5- (Fig. 102E) integrin promoters in a ChIP assay and this was previously observed for the β1-integrin promoter (903).

Figure 102.

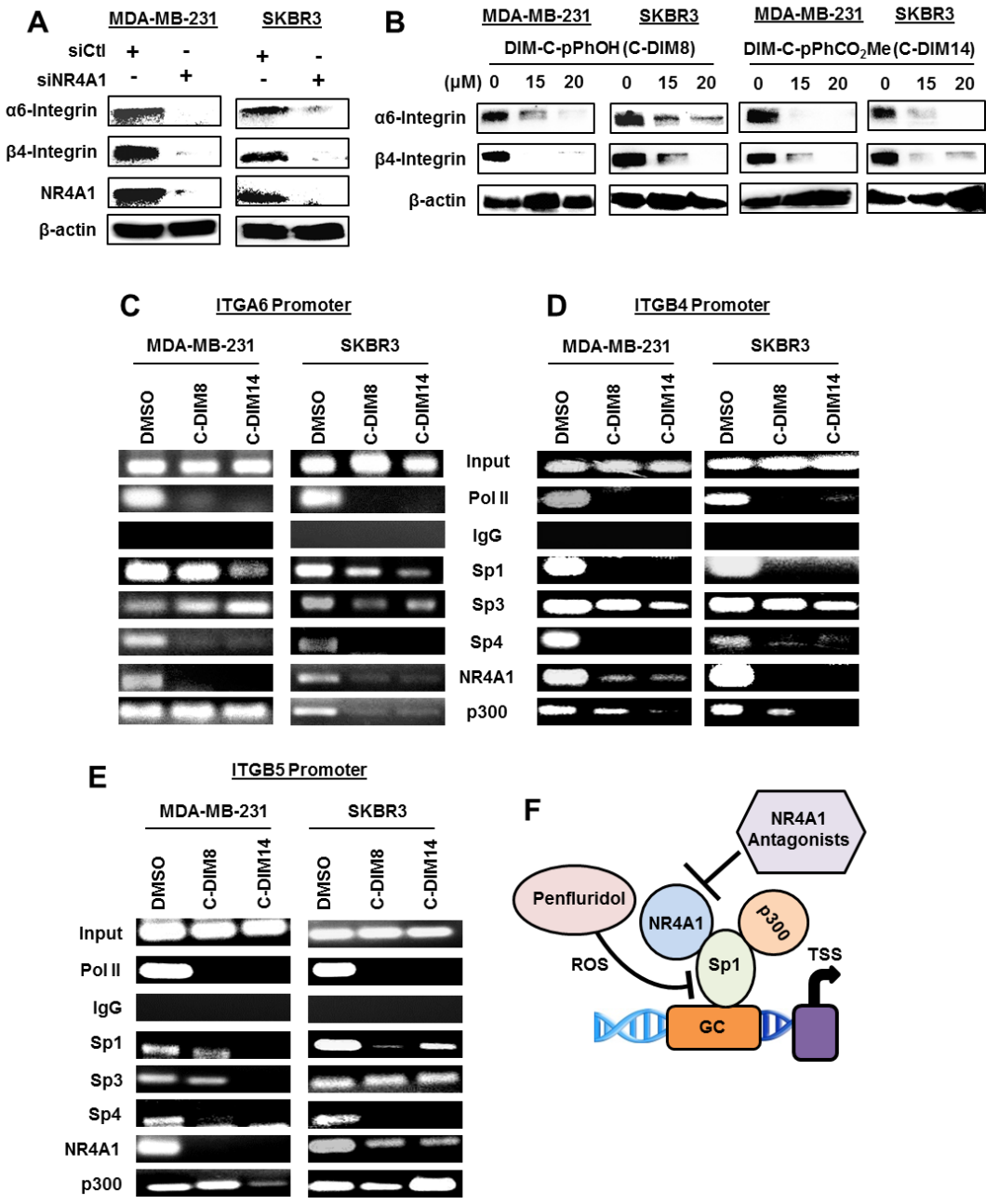

Figure 102. Integrin genes targeted by penfluridol are coregulated by Sp transcription factors and NR4A1. Breast cancer cells were transfected with siNR4A1 (A) or treated with the NR4A1 antagonists DIM-C-pPhOH (C-DIM8) and DIM-C-pPhCO$_2$Me (C-DIM14) (B), and whole cell lysates were analyzed by western blots. Breast cancer cells were treated with DMSO and the two NR4A1 antagonists, and interactions of Sp1, Sp3, Sp4 and other nuclear cofactors with the ITGA6 (C), ITRG4 (D) and ITGA5 (E) gene promoters were determined in a ChIP assay. The same interactions with the ITGB1 promoter were previously reported (11). (F) Model of NR4A1/Sp interactions with GC-rich integrin gene promoters and the differential targeting by penfluridol and NR4A1 antagonists.

Unlike penfluridol which decreased expression of Sp1, Sp3 and Sp4, resulting in loss of these TFs from the integrin promoters (Fig. 101), NR4A1 antagonists or NR4A1 knockdown do not affect Sp expression (903) and their retention or loss from the integrin promoters is highly variable and gene promoter- and cell context-dependent. This variability was observed for p300 (Figs. 102C-102E) and also in our previous report (903). Nevertheless, the results show that both penfluridol and NR4A1 antagonists downregulate expression of α5, α6-, β1- and β-4 integrin by selectively targeting the Sp and NR4A1 transcription factors, respectively, which are required for expression of the integrin genes (Fig. 102F). Treatment of athymic nude mice bearing MDA-MB-231 cells in an orthotopic model with penfluridol (10 mg/kg/day) decreased tumor volume over the treatment period (Fig. 103A) and inhibited tumor growth (Fig. 103B), and toxicity (organ damage or weight loss) was not observed. Western blot analysis of lysates from control (solvent) and penfluridol-treated mice showed that penfluridol decreased expression of Sp1, Sp3 and Sp4, prototypical Sp-regulated genes (survivin and EGFR) including α6-, α5-, β1- and β4-integrin and induced apoptosis (Fig. 103C). These effects were also quantitated (Fig. 103D) and penfluridol significantly decreased or increased (PARP cleavage) compared to control protein set at 100% and normalized to β-actin. Thus, the *in vivo* results complement the *in vitro* studies demonstrating that the anticancer activities of penfluridol are ROS-dependent and this includes NR4A1/Sp-mediated downregulation of integrins.

Figure 103.

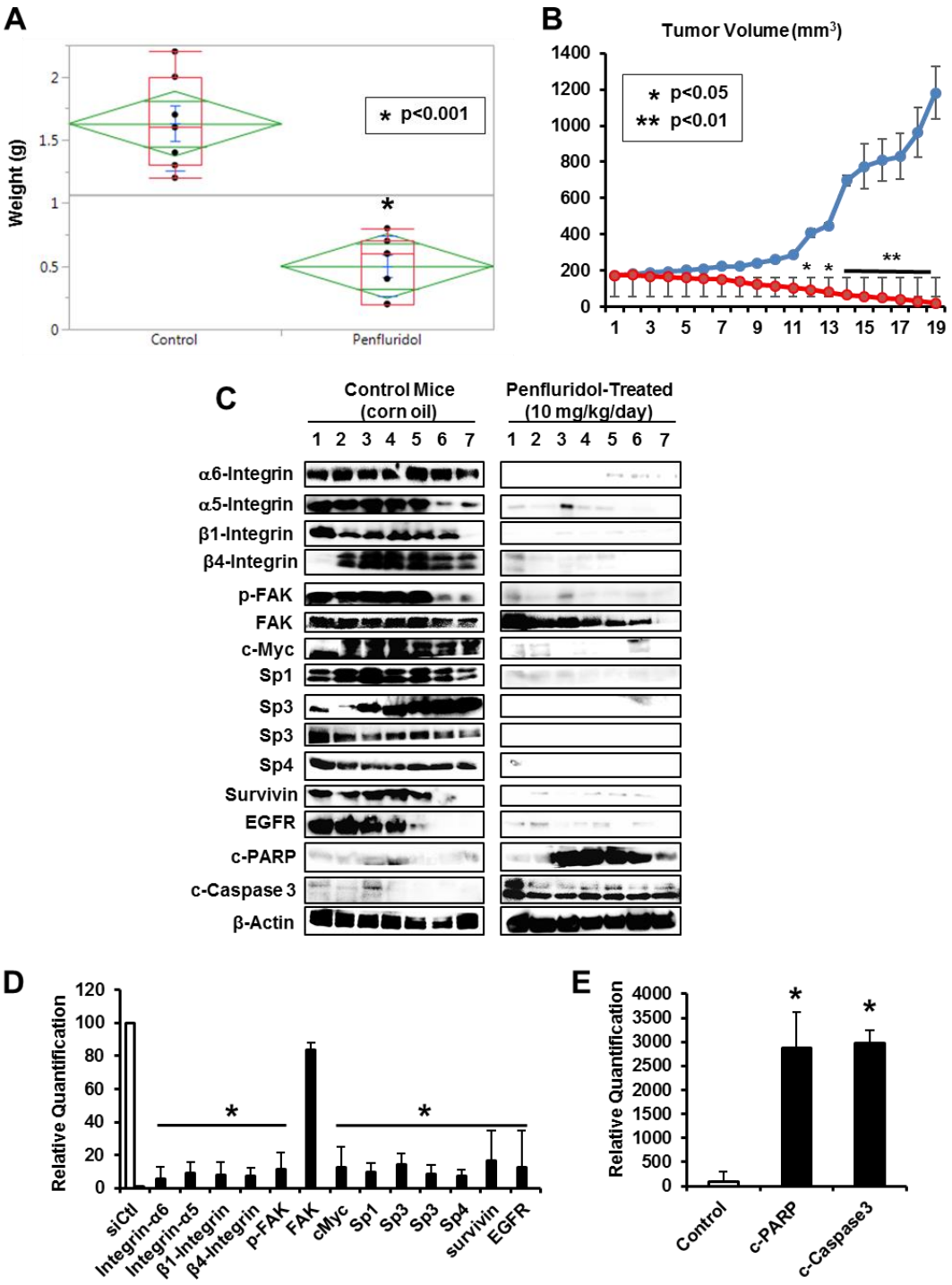

Figure 103. Penfluridol inhibits tumor growth *in vivo*. Penfluridol (10 mg/kg/d) inhibit tumor volume (A) and weight (B) in athymic nude mice bearing MDA-MB-231 cells in an orthotopic model. Western blot analysis of tumor lysates (C) and quantitation of various proteins (D and E) where control proteins normalized to β-actin were set at 100% and penfluridol-mediated changes (normalized to β-action) compared to controls were determined as outlined in the Materials and Methods.

Discussion

Integrins are cell surface receptors that function as α/β heterodimers which are formed from the 18 alpha and 8 beta subunits, and the functions of the known 24 α/β-integrin heterodimers are highly tissue-/cell type-specific. In cancer cells, integrins are important for migration and invasion, and overexpression of some integrins in cancer patients is a negative prognostic factor (841,842). Integrins play a key role in breast cancer stem cells and mammary tumors, and there is extensive evidence for the role of β1-, β4-, α5- and α6-integrins and their α/β heterodimers in breast cancer cell migration, invasion and metastasis (1088-1094). Although integrin inhibitors have been developed, their applications for cancer chemotherapy have been limited (841) and this is due, in part, to β1-β3-integrin switching in which drugs targeting β1-integrin induce β3-integrin (382,1095). We recently showed that the orphan nuclear receptor NR4A1 regulates expression of β1-integrin and treatment of breast cancer cells with bis-indole-derived (C-DIMs) NR4A1 antagonists such as DIM-C-pPhOH and DIM-C-pPhCO$_2$Me decrease both β1- and β3-integrin expression and eliminate the switching pathway (903). Both integrins are regulated by an NR4A1-Sp complex which binds to GC-rich regions of their gene promoters (Fig. 102F), suggesting that β1- and β3-integrin can be targeted by NR4A1 antagonists or drugs that target Sp transcription factors.

The report that penfluridol decreases expression of α6- and β4-integrin (1082), coupled with previous reports showing that Sp proteins interact with the GC-rich regions of the α6- and β4-integrin gene promoters (1085,1086), suggests that the mechanism of action of penfluridol may be due to repression of Sp proteins. We investigated α5-integrin expression which also has a GC-rich gene promoter (1087). Using SKBR3 and MDA-MB-231 cells as a model, it was clear that penfluridol induced ROS and the resulting inhibition of cell growth and migration and induction of apoptosis were all inhibited after cotreatment with GSH (Fig. 97). Subsequent experiments show that induction of ROS was the key factor in mediating downregulating Sp1, Sp3 and Sp4 through ROS-dependent epigenetic repression of cMyc, decreased expression of cMyc-regulated miR-27a and miR-20a/miR-17, and induction of the Sp repressors ZBTB10 and ZBTB4. This ROS-dependent pathway has previously been observed for other ROS inducers such as PEITC and HDAC inhibitors (648,1083). The ROS-miR-ZBTB-Sp pathway (Fig. 99A) has also been observed in cancer cells treated with a nitro-aspirin derivative, celastrol, betulinic acid and curcumin; other ROS inducers including arsenic trioxide, ascorbate, hydrogen peroxide and *t*-butyl hydroperoxide also downregulate Sp1, Sp3 and Sp4 in cancer cell lines (645,648,769,981,983,1000,1084). We also observed that penfluridol targeted Sp TFs and pro-oncogenic Sp-regulated genes *in vivo* and this was consistent with potent inhibition of tumor growth in an orthotopic model (Fig. 103).

This paper also demonstrates that penfluridol-mediated downregulation of α5-, α6-, β1 and β4-integrins is also ROS-dependent and Sp TFs regulate expression of these genes in breast cancer cell lines. Moreover, like β1- and β3-integrin (903), the α5-, α6- and β4-integrins are coregulated by NR4A1 and expression of this family of integrins is due to interactions of the NR4A1-Sp complex with their corresponding GC-rich gene promoter elements (Fig. 102G). Penfluridol does not exhibit NR4A1 antagonist activity but targets the integrins through repression of Sp TFs, whereas the C-DIM/NR4A1 antagonists inactivate the coactivator-like activity of NR4A1 (Fig. 102F). Nuclear receptor-mediated activation of genes through interaction with DNA bound Sp TFs has been observed for several other receptors (1096) and like the integrins in this study can be targeted through inactivation of the receptor or downregulation of Sp TFs. The identification of the mechanism of action of penfluridol coupled with the reported effectiveness of this compound as an inhibitor of breast cancer metastasis will facilitate the design of future clinical applications of this ROS inducer for breast cancer therapy.

CHAPTER IX

MECHANISM OF NR4A1 REGULATION OF TGF-β-INDUCED MIGRATION AND EPITHELIAL TO MESENCHYMAL TRANSITION IN TRIPLE NEGATIVE BREAST CANCER CELLS

Introduction

Transforming growth factor β (TGFβ) signaling plays an important and complex role in cancer and exhibits both tumor-promoting and tumor-suppressor functions that are dependent on the cancer cell context (1097-1099). For example, loss of TGFβ in immortalized but non-tumorigenic MCF-10A cell subtype resulted in enhanced growth due to a less differentiated phenotype and this was accompanied by a decreased population of early progenitor cells (1100). In contrast, TGFβ stimulates later stage cancer cells to migrate and invade and this is linked to induction of epithelial to mesenchymal transition (EMT) and a more aggressive cancer cell phenotype (1097, 1101,1102). TGFβ signaling is initiated by ligand-dependent TGFβ receptor activation which results in formation of an activated SMAD2/SMAD3-SMAD4 nuclear transcription factor complex (1103). In contrast, SMAD7 functions as an inhibitory SMAD by recruiting factors leading to proteasome-dependent degradation of TGFβ receptor 1 (TGFBR1) (1104,1105).

Arkadia, RNF12 and Axin2 also play key roles in activation of TGFβ signaling by inducing polyubiquitination and degradation of inhibitory SMAD7 (1105,1106), and a recent study demonstrated that the orphan nuclear receptor NR4A1 (Nur77, TR3) also has a critical role in SMAD7 degradation (822). It was reported that NR4A1 induces Axin2 expression, and NR4A1 directly interacts with Axin2 and SMAD7 and is an obligatory factor for activation of SMAD7 degradation. This model was derived from studies on inflammatory cytokine-induced MDA-MB-231 breast cancer cell invasion and showed that cytokine-induced NR4A1 was necessary for SMAD7 degradation and TGFβ-induced migration/invasion.

Studies in this laboratory have investigated NR4A1-dependent pro-oncogenic pathways in breast and other cancer cell lines and have developed 1,1-bis(3-indolyl)-1-(*p*-substituted phenyl) methane (C-DIM) NR4A1 ligands that act as NR4A1 antagonists in cancer cell lines and tumors (788,803-806,812,904,903). In MDA-MB-231 breast cancer cells, we demonstrated that basal migration/invasion is dependent on NR4A1-regulated expression of β1-integrin which can be inhibited by C-DIM/NR4A1 antagonist (903). TGFβ markedly enhances MDA-MB-231 cell migration/invasion and, as previously reported (822), we observed that this response is NR4A1-dependent and could also be inhibited by C-DIM/NR4A1 antagonists. Our studies demonstrated that the key inhibitory effect of the NR4A1 antagonist is to block nuclear export of NR4A1 and thereby prevent interactions with the cytosolic Axin2/RNF12/Arkadia-SMAD7 complex.

In this study, we have further investigated the critical and essential role of NR4A1 in regulating TGFβ-induced migration/invasion and show that TGFβ-induced nuclear export of NR4A1 is dependent on activation of the p38MAPK pathway. We also show that TGFβ induces expression of both NR4A1 and β-catenin, and these responses are also p38-dependent. However, induction of NR4A1 is dependent on β-catenin and its interactions with LEF1/TCF3 and TCF4 bound to the NR4A1 promoter. These studies define a unique role for NR4A1 in TGFβ-induced migration and EMT in estrogen receptor (ER)-negative breast cancer cells and also demonstrate that NR4A1 antagonists block this response, indicating potential clinical applications for treatment of both early and late stages of this disease.

Materials and Methods

Cell lines, reagents, and antibodies

Breast cancer (MDA-MB-231, H5887T, and SUM159) were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained 37˚C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic. NR4A1, arkadia, immunofluoresent-grade arcadia (H00054778-M05) and RNF12 antibodies was purchased from Novus Biologicals (Littleton, CO). TGF-β was purchased from BD Biosystems (Bedford, MA). β-actin antibody, Dulbecco's Modified Eagle's

Medium, M2 Flag antibody, MG132 and 36% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Hematoxylin was purchased from Vector Laboratories (Burlingame, CA). β-catenin, GAPDH, p38, p-p38, p-NR4A1, Axin2, Anti-rabbit IgG Fab2 Alexa Fluor® 488 or Anti-mouse IgG Fab2 Alexa Fluor®, phalloidin (Alexa Fluro® 555 Phalloidin), leptomycin B, SP600125, SB202190, LY294002, PD98059 NR4A1 immunofluorescent antibody were purchased from Cell Signaling Technologies (Manassas, VA) Smad 6/7 and ubiquitin antibody from Santa Cruz Biotech (Santa Cruz, CA); p84 antibody from GeneTex (Irvine, CA).

Plasmids

FLAG-NR4A1, FLAG-NR4A1-(A-B), and FLAG-NR4A1-(C-F) were synthesized in the lab using site directed mutagenesis. DDK-Myc-p38CA-(D176A; F327S), DDK-Myc-p38KD-(K53A), DDK-Myc-p38DN-(T180A;Y182F), DDK-Myc-arkadia, , DDK-Myc-arkadia-(Δ405-989), DDK-Myc-arkadia-(Δ1-404), DDK-Myc-arkadia- (241-404), and DDK-Myc-arkadia (C937A) were purchased from Origene Technologies (Rockville, MD). Plasmids were transfected as described previously (891,892,910,941,942), media was removed and then cells were treated with respective compound.

Boyden chamber assay

MDA-MB-231, SUM159, and H5587T cancer cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium

supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. Cells were seeded and subsequently treated with varying concentration of DIM-C-pPhOH or DIM-C-pPhCO$_2$Me for 24 hours or 1 hr prior (+/- TGFβ [5ng/ml] 4 h cotreatment) or with 100 nm of siAxin2, siArkadia, siRNF12, siβ-catenin, siTAK1, siMKK3, siMKK6, sip38α, siTCF1, siTCF3, siTCF4, or siLEF1 for 48 hours. Cells were trypsinized, counted then placed in 24-well 8.0μm pore ThinCerts from BD Biosciences (Bedford, MA) allowed to migrate for 24 hr, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted as described (891,892,910,941,942).

RT PCR

RNA was isolated using Zymo Research *Quick-RNA* MiniPrep kit (Irvine, CA). Quantification of mRNA (slug, snail, and NR4A1) was performed using Bio-Rad iTaq Universal SYBER Green 1-Step Kit (Richmond, CA) using the manufacturer's protocol with real-time PCR. TATA Binding Protein (TBP) mRNA was used as a control to determine relative mRNA expression. The following primers were used: Slug: Forward primer: 5′ AGCAGTTGCACTGTGATGCC 3′, Reverse primer: 5′ ACACAGCAGCCAGATTCCTC 3′; Snail: Forward primer: 5′ TGCCCTCAAGATGCACATCC 3′, Reverse primer: 5′ TGACATCTGAGTGGGTCTGC 3′; NR4A1: Forward primer 5' TCCCATATTGGGCTTGGATA 3', Reverse primer: 5' ATCTTGGGATTCTCCCTTCG 3'.

Immunoprecipitation

MDA-MB-231 cancer cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or TGFβ(5 ng/ml) was added for 4 hr (after +/- pretreatment with leptomycin B (20 nM) for 24 hr or +/- pretreatment with +/- DIM-C-pPhOH (20 µM), DIM-C-pPhCO$_2$Me (15 µM), SB202190 (30µM) for 1 hr., or transfection of DDK-Myc-p38CA, DDK-Myc-p38KD, DDK-Myc-p38DN, D-NR4A1, FLAG-NR4A1-(A-B), FLAG-NR4A1-(C-F), DDK-Myc-arkadia, DDK-Myc-arkadia (Δ405-989), DDK-Myc-arkadia (Δ1-404), DDK-Myc-arkadia (241-404), and DDK-Myc-arkadia (C937A) plasmids 6 hr. post transfection. Protein A Dynabeads were prepared and binding of antibody with protein and protein-protein interactions were isolated by Life technologies Immunoprecipitation Kit using Dynabeads coated with Protein A (Grand Island, NY) following manufacturer's protocol. Protein-protein interactions of interest were determined using western blot.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. MDA-MB-231 cells were

treated with DMSO or TGFβ (5 ng/ml) was added for 4 hr. (after +/- pretreatment with leptomycin B (20 nM) for 24 hr. or +/- pretreatment with +/- DIM-C-pPhOH (20 μM), DIM-C-pPhCO$_2$Me (15 μM), SB202190 (30μM) for 1 hr., or DDK-Myc-p38CA, DDK-Myc-p38KD, DDK-Myc-p38DN 6 hr. post transfection. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (~200 to 1,500 bp). The sonicated chromatin was immunoprecipitated with normal IgG, p300 (Santa Cruz), siSp1 (Abcam), NR4A1 (Novus Biologicals), or RNA polymerase II (pol II; Active Motif) antibodies and protein A-conjugated magnetic beads at 4°C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. DNA was prepared by proteinase K digestion followed by PCR amplification. The primers for detection of the NR4A1 promoter region were 5′- CGAGGAGCCTATTTATAG -3′ (sense) and 5′- TCGACGTTTGGCCATACAAGG -3′ (antisense) PCR products were resolved on a 2% agarose gel in the presence of RGB-4103 GelRed Nucleic Acid Stain.

Nuclear/cytosolic extraction

MDA-MB-231 cancer cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. The medium was

then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or TGFβ(5 ng/ml) was added for 4 hr. (after +/- pretreatment with leptomycin B (20 nM) for 24 hr. or +/- DIM-C-pPhOH (20 μM), DIM-C-pPhCO_2Me (15 μM), SP600125 (30μM), SB202190 (30μM), LY294002 (30μM) or PD98095 (30μM) for 1 hr., or transfection of p38CA, p38KD, or p38DN plasmids 6 hr. post transfection. Nuclear and cytosolic fractions were then isolated using Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (Rockford, IL) according to manufacturer's protocol. Fractions were then analyzed by western blot. GAPDH and p84 were used as cytoplasmic and nuclear positive controls respectively.

Immunofluorescence

MDA-MB-231 (1.0 x 10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or TGFβ(5 ng/ml) was added for 4 hr (after +/- pretreatment with leptomycin B (20 nM) for 24 hr or +/- pretreatment with +/- DIM-C-pPhOH (20 μM), DIM-C-pPhCO_2Me (15 μM), SB202190 (30μM) for 1 hr., or transfection of DDK-Myc-p38CA, DDK-Myc-p38KD, DDK-Myc-p38DN, D-NR4A1, FLAG-NR4A1-(A-B), FLAG-NR4A1-(C-F), DDK-Myc-arkadia, DDK-Myc-arkadia (Δ405-989), DDK-Myc-arkadia (Δ1-404), DDK-Myc-arkadia (241-404), and DDK-Myc-arkadia (C937A) plasmids 6 hr. post transfection. Cells were then treated with fluorescent

NR4A1 primary antibody (Nur77 (D63C5)) XP®), β-catenin primary antibody, or arkadia (H00054778-M05) primary antibody for 24 hrs. Cells were then washed with PBS and then treated with Anti-rabbit IgG Fab2 Alexa Fluor® 488 or Anti-mouse IgG Fab2 Alexa Fluor® secondary antibody for 3 hrs. Cells were then treated with DAPI (Biotium, Hayward, CA) and phalloidin (Alexa Fluro® 555 Phalloidin) for 15 min in the dark following manufacturers protocol. After washing with ice cold PBS cells were visualized by microscope (Advanced Microscopy). NR4A1, β-catenin, or arcadia localization was determined by green fluorescence (GFP filter). DAPI was used to stain the nucleus and phalloidin stained F-actin (rhodamine filter). Images were taken sequentially of NR4A1 β-catenin, or arkadia, phalloidin, and DAPI, then merged.

Western blot analysis

MDA-MB-231, SUM159, and H5587T cancer cells (3.0 x 105 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. Cells were transfected with 100 nm of siAxin2, siArkadia, siRNF12, siβ-catenin, siTAK1, siMKK3, siMKK6, sip38α, siTCF1, siTCF3, siTCF4, or siLEF1 for 72 hours or treated with various conditions as described above. Cells were analyzed by western blot as described previously (24-28).Cells were analyzed by western blot as described previously (891,892,910,941,942).

Small interfering RNA interference assay

SiRNA experiments were conducted as described previously (). The siRNA complexes used in the study are as follows: siGL2-5': CGU ACG CGG AAU ACU UCG A; siNR4A1: SASI_Hs02_00333289; siAxin2: SASI_Hs01_00110148; siArkadia: SASI_Hs01_00064840; siRNF12:SASI_Hs01_00238255; siTAK1: SASI_Hs02_00335227; siMKK3: SASI_Hs01_00228024; siMKK6: SASI_Hs01_00162806; sip38α: SASI_Hs01_00018467; siβ-catenin: SASI_Hs02_00318698; siTCF1: SASI_Hs01_00018982; siTCF3: SASI_Hs01_00214771; siTCF4: SASI_Hs02_00317381; siLEF1: SASI_Hs02_00349169

Statistical analysis

Statistical significance of differences between the treatment groups was determined as .previously described (891,892,910,941,942).

Results

TGFβ-dependent inhibition of migration is dependent on activation of p38-mediated nuclear export of NRA41

It was initially reported that TGFβ-induced EMT and migration/invasion were dependent on NR4A1 which formed an E3 ligase-Axin2/RNF12/Arkadia complex that degraded SMAD7 and thereby activated TGFβR1 (822). Subsequent studies in this laboratory indicated that a key step in this pathway involved TGFβ-induced nuclear export of NR4A1 (903), and the mechanisms of TGFβ-NR4A1 crosstalk were further investigated in ER-negative breast cancer cells. Results in Figure 1A show that among a series of kinase inhibitors, LY294002 and PD98059 inhibited basal migration of MDA-MB-231 cells, but only the p38MAPK inhibitor SB202190 significantly inhibited TGFβ-induced migration of MDA-MB-231 cells. We also observed that the p38 inhibitor SB202190 also inhibited TGFβ-induced migration in H5587T and SUM159 triple negative breast cancer cells (Fig. 104A), whereas LY294002 and PD98059 decreased basal but not TGFβ-induced migration as observed in MDA-MB-231 cells. TGFβ induces expression and nuclear export of NR4A1 (903), and we now show that this response was significantly inhibited in MDA-MB-231 cells cotreated with the p38 inhibitor SB202190 (Fig. 104B). In contrast, SP600125, LY294002 and PD98059 did not inhibit TGFβ-dependent nuclear export of NR4A1; however, the latter two inhibitors decreased induction of NR4A1 by TGFβ (Fig. 104B).

These results suggest that TGFβ induces activation of p38, and this was confirmed in MDA-MB-231 cells where treatment with TGFβ enhanced phosphorylation of p38a T180/Y182 and also increased phospho-NR4A1 (S351) expression (Fig. 104B). TGFβ also induced NR4A1 expression and nuclear export

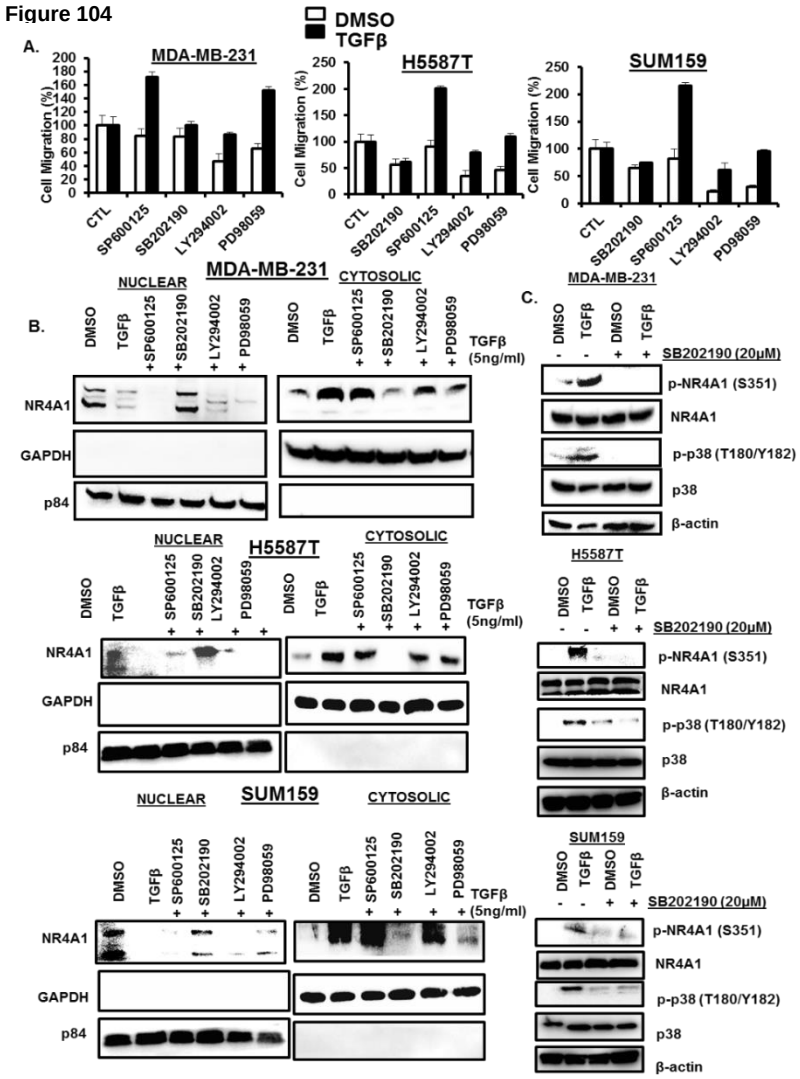

Figure 104. Effects of kinase inhibitors on TGF-β/NR4A1 dependent cell migration. The p38 inhibitor SB02190 inhibited TGFβ-induced migration (A) and NR4A1 nuclear export (B) whereas the c-JNK inhibitor SP600125, PI3K inhibitor LY294002, and the MEK inhibitor PD98059 did not. TGFβ also induced migration in H5587T and SUM 159 breast cancer cell lines and NR4A1 nuclear export and p38 inhibition also blocked these effects. C) TGFβ induced phosphorylation of NR4A1 (S355) which was inhibited by SB202190 and this was observed in all the three cell lines.

in H5587T and SUM159 cells (Figs. 104C and 104D) and this was inhibited by SB202190. SP600125, LY294002 and PD98059 did not inhibit TGFβ-mediated nuclear export of NR4A1 but enhanced expression of the receptor. TGFβ also induced phosphorylation of NR4A1 (S351) and p38 (T180/Y182) in H5587T and SUM159 cells, and SB202190 inhibited this response (Figs. 104C and 104D), confirming the TGFβ-induced NR4A1 nuclear export was p38-dependent in the three ER-negative breast cancer cell lines.

The role of p38 in mediating TGFβ-induced nuclear export of NR4A1 was further ınvestıgated using constructs expressing constitutively active [p38(CA)], kinase domain inactive [p38(KD)], and dominant negative p38 [p38(DN)] constructs in MDA-MB-231 cells. p38(KD) and p38(DN) slightly increased expression of nuclear NR4A1 but did not induce nuclear export of the receptor, whereas both p38(CA) and TGFβ induced expression and nuclear export of NR4A1 protein (Fig. 105A). Figure 105B illustrate that p38(CA) alone and in combination with TGFβ induced expression and nuclear export of NR4A1, whereas p38(KD) and p38(DN) alone and in combination with TGFβ did not induce expression or nuclear export of the receptor, demonstrating that TGFβ did not rescue/enhance nuclear export of NR4A1 in cells expressing p38(KD) or p38(DN). Previous studies showed that the nuclear export inhibitor LMB and NR4A1 antagonists CDIM8 [1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH)] and CDIM14 [1,1-bis(3'-indolyl)-1-(*p*-

carboxymethylphenyl)methane (DIM-C-pPhCO$_2$Me)] blocked TGFβ-induced nuclear export of NR4A1 (903). In this study, these same treatments plus LMB/TGFβ also inhibited p38(CA)-dependent nuclear export of NR4A1 (Fig. 105C). Overexpression of p38(CA) but not p38(KD) or p38(DN) induced MDA-MB-231 cell migration after 5 and 12 hr (Fig. 105D) and the combination of p38 and TGFβ did not induce cell migration greater than that observed for each treatment alone (Fig. 105F). Both p38(KD) and p38(DN) alone did not induce MDA-MB-231 cell migration and in combination with TGFβ, this inhibitory effect was not rescued, indicating that both kinases inhibited TGFβ-induced migration. Results in Figure 105E and 105F demonstrate that the combination of TGFβ + p38(CA) did not enhance MDA-MB-231 cell invasion, and we also show that in combination with treatments (LMB, CDIM8, CDIM14 and SB202190) that inhibit nuclear export of NR4A1 (Figs. 104B and 104C), there was also inhibition of p38/TGFβ-induced invasion (Fig. 105F). We also used immunostaining and microscopy to investigate treatment-related cytosolic and nuclear localization of NR4A1. Results demonstrate that p38CA and TGFβ treatment induced nuclear export (Figure 3A) and this export was significantly abrogated by pretreatment with SB202190, leptomycin B, C-DIM 8, and C-DIM 14 (Figure 106A). Both p38(KD) and p38(DN) alone did not induce NR4A1 nuclear export and supplementation with TGFβ did not rescue this effect (Fig. 106B), corroborating results obtained from Figures 105A and 105B.

Figure 105.

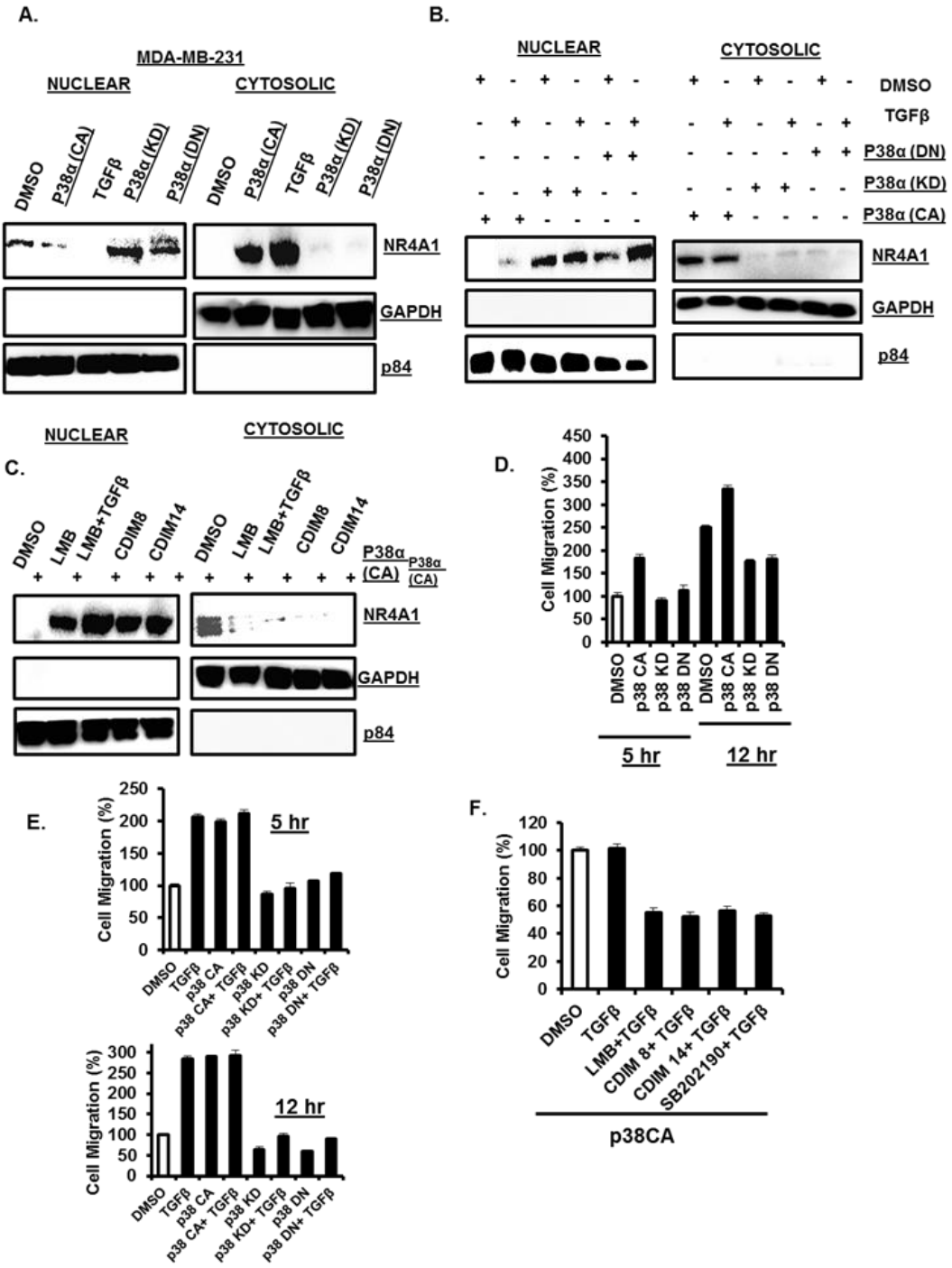

Figure 105. p38α induces TNBC cell migration and NR4A1 nuclear export. Transfection of p38α (CA) induced NR4A1 nuclear export (A) and migration (D,E) whereas transfection of p38α (KD) and p38α (DN) failed to induce nuclear export (A,B) or induce migration (D,E) and this effect was not rescued by TGFβ (B,E). Transfection of p38α (CA) and cotreatment with LMB, LMB+TGFβ, or NR4A1 antagonists inhibited this nuclear export (C) and migration (F)

Figure 106.

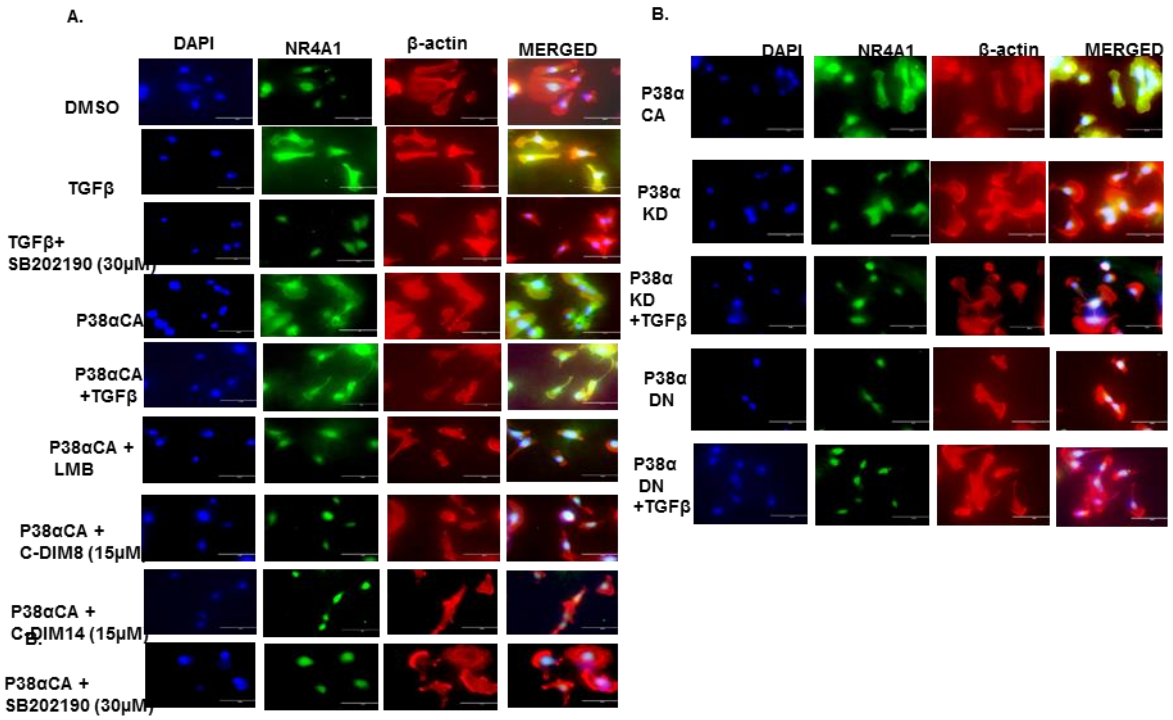

Figure 106: NR4A1 localization by TGF-β,p38α, and C-DIMs. Transfection of p38α (CA) induces NR4A1 nuclear export (A) which is blocked by cotreatment with LMB, C-DIM/NR4A1 antagonists and SB202190. Transfection of p38α (KD) or p38α (DN) failed to induce nuclear export of NR4A1 (B) and TGFβ did not rescue this phenotype.

NR4A1 interactions with Axin2, NRF12, Arkadia and SMAD7

It was previously reported that NR4A1 played a role in Axin2-RNF12/Arkadia-induced SMAD7 degradation (822), and in this study we investigated the interactions of NR4A1 with proteins required for that activity.

MDA-MB-231 cells were treated with 5 ng/ml TGFβ for 4 hr, and cell protein lysates were immunoprecipitated with NR4A1 antibodies. Western blots of the immunoprecipitated protein showed that NR4A1 was associated with Axin2, Arkadia, RNF12 and SMAD7 (Fig. 107A). NR4A1 interactions with this cytosolic protein complex were abrogated after treatment with the NR4A1 ligands CDIM8, CDIM14, LMB alone and in combination with TGFβ (Fig. 107A). These latter treatments maintained NR4A1 in the nucleus (903), suggesting that NR4A1 interacts with these proteins in the cytosol. We also examined interactions of the C- and N-terminal regions of N4RA1 with Axin2/RNF12/Arkadia/SMAD7 complex by transfecting cells with FLAG-NR4A1(C-F) or FLAG-NR4A1(A-B) and immunoprecipitated protein lysates with FLAG antibodies (Fig. 107B and 107C). The C-terminal domain [FLAG-NR4A1(C-F)] interacted with Axin 2 and Arkadia and to a lesser extent RNF12 (Fig. 4B), whereas TGFβ and p38CA induced interactions of the N-terminal domain of NR4A1 [FLAG-NR4A1(A-B)] with Axin2 and SMAD7 (Figs. 107C and 107D) and these interactions were abrogated by leptomycin B, C-DIM 8, C-DIM 14, and SB202190 (Fig. 107D).

Moreover, association of NR4A1 with the complex was not observed after treatment with p38KD or p38DN alone or supplemented with TGFβ (Figs 107D and 107F). We also observed in MDA-MB-231 cells transfected with FLAG-NR4A1(full length) that FLAG antibodies co-immunoprecipitated Axin2, Arkadia, RNF12 and SMAD7 (Fig. 107E) only in cells transfected with p38(CA) but not p38(KD) or p38(DN). Transfection of MDA-MB-231 cells with FLAG-NR4A1(A-B)

(Fig. 107C) or FLAG-NR4A1(C-F) (Fig. 107E) and overexpression of p38(CA) (± TGFβ) followed by immunoprecipitation with FLAG antibodies demonstrates that the C-terminal domain of NR4A1 interacts with Axin2, Arkadia, RNF12 and SMAD7 (Fig. 107D) and the N-terminal domain interacts with only SMAD7 and Axin2 (Fig 107G). In contrast, p38(KD) or p38(DN) ± TGFβ did not induce interactions of NR4A1 with these proteins (Figs. 107D, 107E, 107F), suggesting that both p38 mutants inhibited the TGFβ-induced response. Similar results were obtained with cells transfected with DDK-Myc-arkadia under same treatment conditions (Suppl. Fig. A-16). These results demonstrate that NR4A1 export is important for interactions with the ubiquitination complex and the C-terminal domain of NR4A1 is necessary for interactions with all members of this complex. The C-DIM/NR4A1 antagonists and the p38 inhibitor block NR4A1 nuclear export and interactions with the ubiquitination complex proteins.

Figure 107.

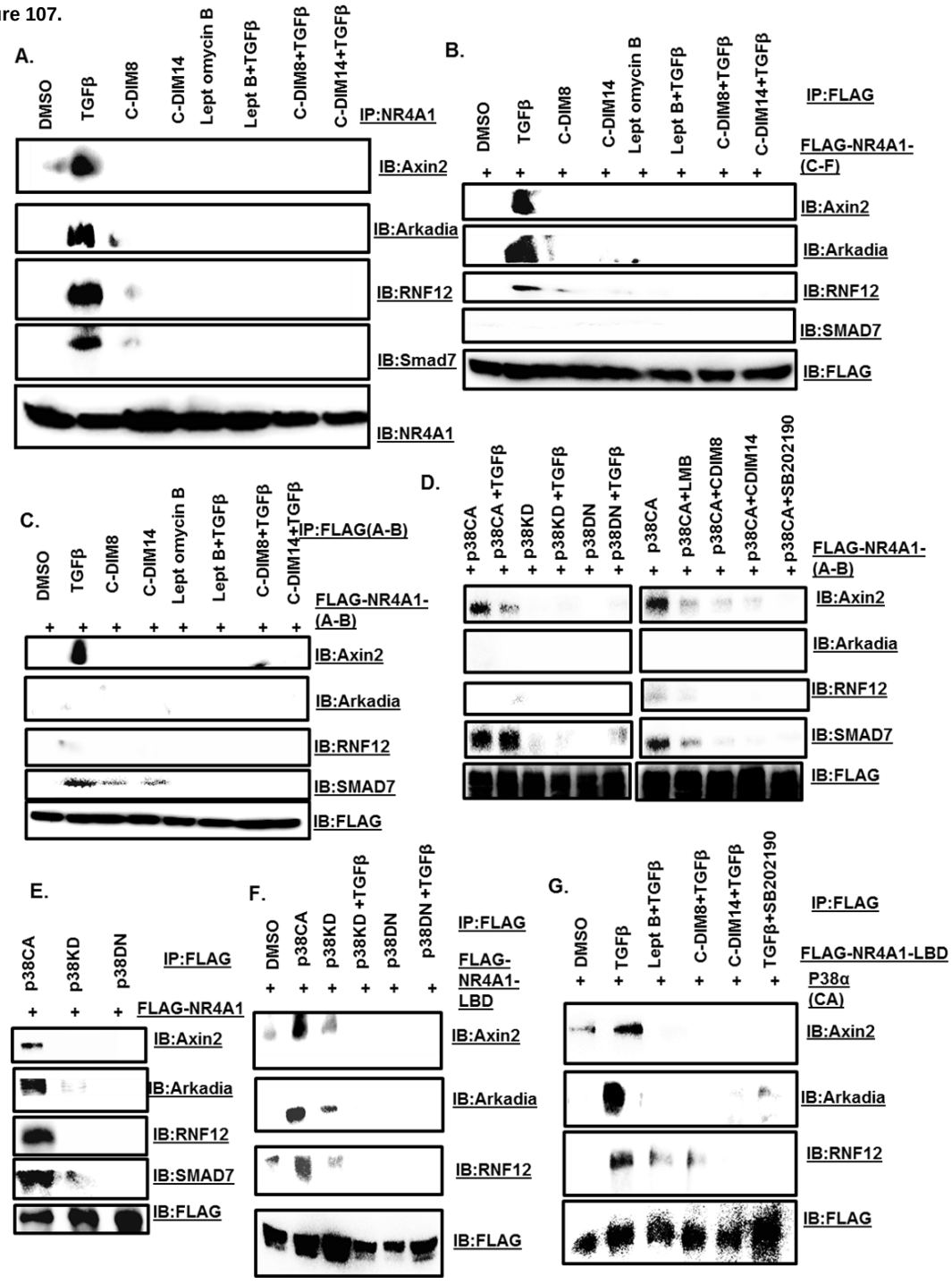

Figure 107: Interactions of NR4A1 with ubiquitination complex. Treatment with TGFβ (A) or p38α (CA) (E) induces association of NR4A1 with Axin2, Arkadia, RNF12, and SMAD7 (A) and this was inhibited by C-DIM/NR4A1 antagonists, LMB and SB202190. SMAD7 and Axin2 binds to NR4A1-(A-B) (C,D) whereas RNF12, Arkadia, and Axin2 bind to NR4A1-(C-F) (B,F,G). Transfection with p38α (KD) or p38α (DN) did not induce association (E) and TGFβ could not rescue this phenotype (F).

Results illustrated in Figure 108A show that knockdown of Axin2, Arkadia and RNF12, but not SMAD3 or SMAD2, by RNAi inhibit TFGβ-induced migration of MDA-MB-231 cells. This demonstrates that like NR4A1, proteins constituting this ubiquitin ligase complex also play an important functional role in mediating TGFβ-induced cell migration and this may be due, in part, to their interactions with NR4A1 to form an active ubiquitin ligase complex. However, it is also possible that decreased migration may be due to changes in TGFβ-induced nuclear export of NR4A1 or other proteins. Therefore, MDA-MB-231 cells were treated with TGFβ and transfected with oligonucleotides that knockdown Axin2 (siAxin2), Arkadia (siArkadia) and RNF12 (siRNF12). TGFβ induced nuclear export of NR4A1 and this was only partially inhibited by siAxin2 and significantly blocked by siArkadia and siRNF12 (Fig. 108B) and similar results were observed in cells expressing p38(CA) (Fig. 108C). Thus, knockdown of members of the ubiquitin ligase complex completely (siArkadia and siRNF12) or partially (siAxin2) blocked TGFβ-induced nuclear export of NR4A1 and this is consistent with their inhibition of TGFβ-induced cell migration (Fig. 108A).

Moreover, using the same knockdown protocol coupled with treatment with TGFβ, we also examined interactions of NR4A1 and SMAD7 with Arkadia, Axin2 and RNF12 in immunoprecipation experiments (Figs. 108D and 108E). Knockdown of Axin2, Arkadia and RNF12 dramatically decreased interactions of NR4A1 with all complex proteins but not SMAD7 (Fig. 108D). The fact that Smad7

and NR4A1 interact was surprising as they are in differential compartments upon knockdown of components of destruction complex. This is being further investigated and perhaps can be explained by a percentage of NR4A1 remaining in equilibrium within nuclear and cytosolic compartments. Smad7 may also exhibit such an equilibrium. In contrast, SMAD7 interactions with Arkadia were decreased by siAxin2 and siRNF12 and interaction with RNF12 was decreased by siArkadia, whereas NR4A1 interactions with SMAD7 were unchanged by loss of Ardadia, Axin2 or RNF12 (Fig. 108E). Figure 5F illustrates the knockdown efficiency of siArkadia, siAxin2 and siRNF12 and show that members of this ubiquitin ligase complex do not affect TGFβ-induced NR4A1 expression which is increased after transfection with siArkadia and siRNF12.

Figure 108.

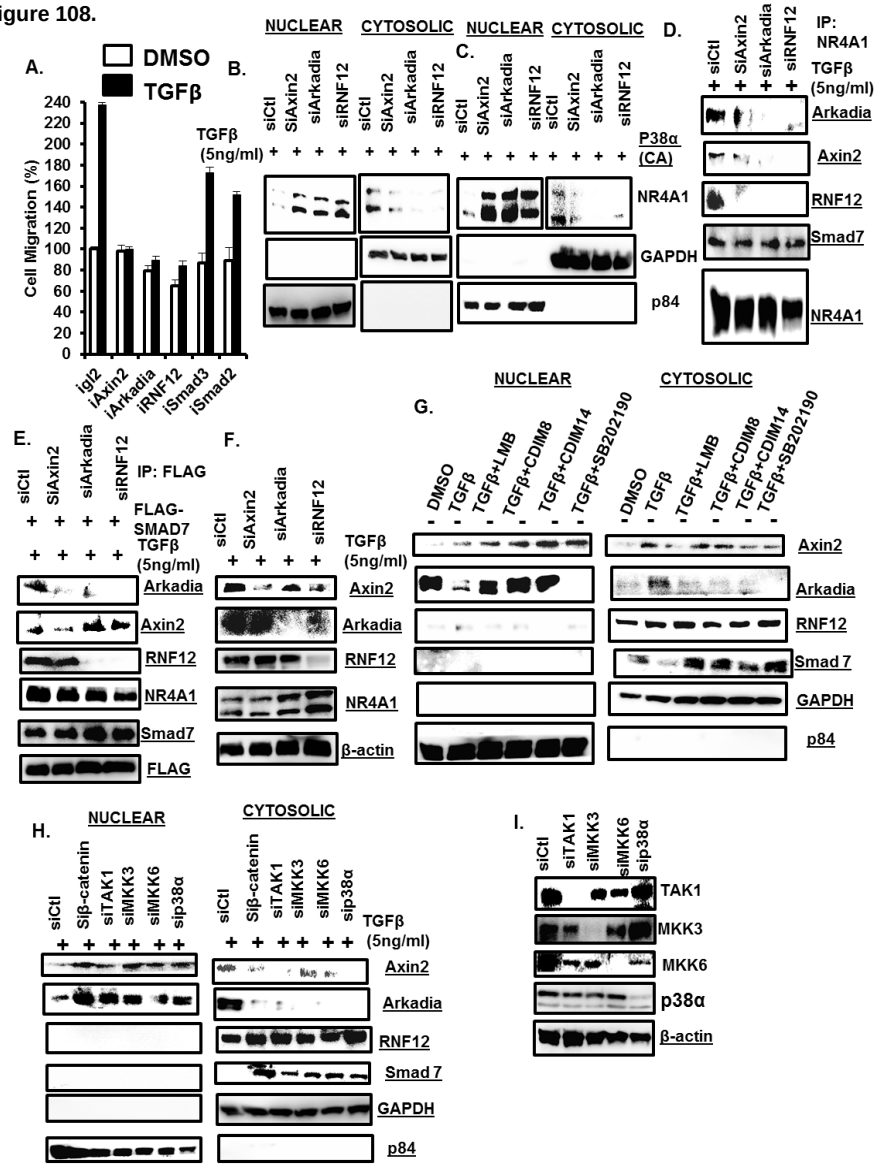

Figure 108: Ubiquitination complex components are required for NR4A1 nuclear export. Knockdown of Axin2, Arkadia, RNF12 (but not SMAD3 or SMAD2) inhibits TGFβ-induced migration (A). Knockdown of Axin2, Arkadia, or RNF12 inhibted TGFβ (B) or p38α(CA) (B) induced nuclear export of NR4A1 and association of the E3 ligase complex (D,E). TGFβ induces nuclear export of Axin2 and Arkadia (G) and this is blocked by LMB, C-DIM/NR4A1 anatgonists, SB202190 or knockdown of kiases upstream of p38α(H). Knockdown efficiency of siAxin2,siArkadia, siRNF12 (F) siTAK1, siMKK3, siMKK6, and sip38α (I) is also demonstrated.

Although the activated ubiquitin ligase complex containing NR4A1 is cytosolic, the constitutive and TGFβ/p38-induced intracellular location of Arkadia,

Axin2 and RNF12 have not previously been determined and were therefore investigated. TGFβ alone induced Axin2 which was detected in both the nuclear and cytosolic fraction and cotreatment with inhibitors of NR4A1 nuclear export (LMB, CDIM8, CDIM14 and SB202190) did not affect expression or location of Axin2 (Fig. 108G). In contrast, Arkadia expression was primarily nuclear and TGFβ-induced nuclear export of Arkadia was inhibited after cotreatment with LMB, CDIM8 and CDIM14. This pattern of TGFβ-induced nuclear export and inhibition for Arkadia was similar to that observed for NR4A1 and since these proteins interact (Fig. 107A), they may be acting as co-chaperones. We also examined the effects of p38 and upstream kinases on the subcellular distribution of the ubiquitin ligase complex proteins by knockdown of p38 (sip38) and upstream kinases TAK1 (siTAK1), MKK3 (siMKK3) and MKK6 (siMKK6) (Fig. 108H). Axin2 was both nuclear and cytosolic for most treatments; however, sip38 and siTAK1 decreased levels of cytosolic Axin2. TGFβ induced nuclear export of Arkadia as observed in Figure 5G; however, this induced response was inhibited by knockdown of p38 and upstream kinases (TAK1, MKK3 and MKK6). Figure 5I illustrates the knockdown efficiency of siTAK1, siMKK3, siMKK6, and sip38α RNF12 and SMAD7 were primarily cytosolic and were unaffected by the treatments. These results confirm that TGFβ-induced p38 and upstream kinases were also important for Arkadia (and NR4A1) nuclear export which is necessary for formation of the active cytosolic ubiquitin ligase complex and TGFβ-induced invasion of MDA-MB-231 cells.

SMAD7 as a target of TGFβ-induced NR4A1 nuclear export

TGFβ-induced degradation of SMAD7 is a key event for migration of breast cancer cells (822) and therefore, we investigated the role of NR4A1 and NR4A1 nuclear export in mediating SMAD7 degradation. SMAD7 is expressed in MDA-MB-231 cells (Fig. 109A) and treatment with TGFβ decreased SMAD7. However, treatment with TGFβ plus agents that inhibit nuclear export of NR4A1 (LMB, CDIM8 and CDIM14) or p38 activity (SB202190) blocked proteasome-dependent downregulation of SMAD7, and the proteasome inhibitor MG132 also blocked the TGFβ-induced response. Moreover, TGFβ and p38(CA) alone or in combination also decreased SMAD7 protein and this was blocked by MG132, whereas p38(KD) and p38(DN) alone or in combination with TGFβ did not affect SMAD7 expression (Fig. 109B). Thus, treatment with TGFβ did not overcome or rescue the cells from the effects of p38(KD) or p38(DN). These results show that TGFβ- and p38-induced nuclear export of NR4A1 are necessary for activating the E3 ubiquitin ligase complex (Arkadia, NR4A1, RNF12 and Axin2) and for proteasome-dependent degradation of SMAD7. This was also confirmed by examination of SMAD7 ubiquitination. Inhibitors that blocked nuclear export of NR4A1 (Fig. 109C) and inactivation of p38 (Figs. 109D and 109E) all inhibited TGFβ-induced ubiquitination of SMAD7 which appears as a streaking band in the gel which is typical of blots containing multiple polyubiquitinated proteins. In addition, we also show that TGFβ-induced ubiquitination is partially blocked by

Axin2 knockdown and completely inhibited by knockdown of Arkadia and RNF12 (Fig. 109F), demonstrating the importance of members of the ubiquitin ligase complex in mediating ubiquitination and subsequent degradation of SMAD7.

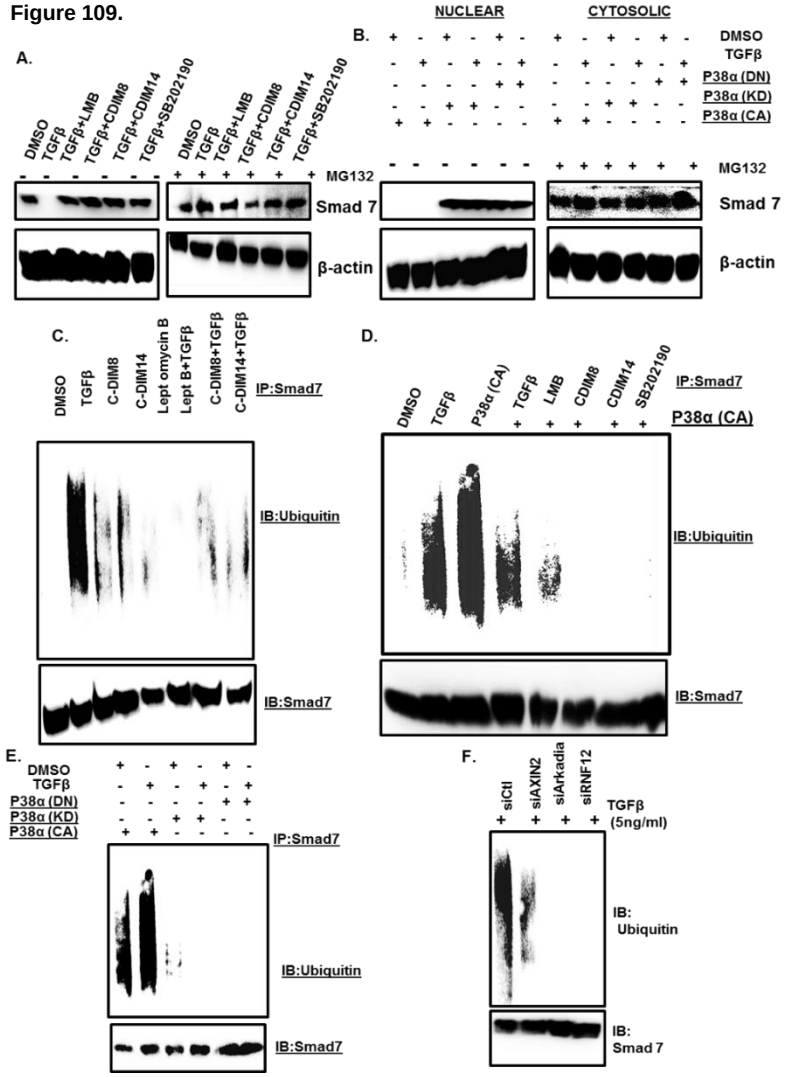

Figure 109: SMAD7 as a target of TGF-β-induced NR4A1 nuclear export. Treatment with TGFβ (A) or p38α(CA) (B) induced SMAD7 degradation and this phenotype was rescued by pretreatment with C-DIM/NR4A1 antagonists, LMB,SB202190, or proteasome inhibitor MG132. Transfection of p38α (KD) or p38α (DN) failed to induce SMAD7 degradation (B). TGFβ and p38α (CA) also promoted poly-ubiquitination of SMAD7 which was abrogated by C-DIM/NR4A1 antagonists, LMB, and SB202190. Knockdown of Axin2, Arkadia, and RNF12 also inhibited this effect (F) and transfection of p38α(KD) and p38α(DN) did not induce poly-ubiquitination of SMAD7 and this was not rescued by cotreatment with TGFβ

NR4A1 plays a role in TGFβ-induced β-catenin and EMT

Previous studies show that p38 induces expression of β-catenin and results illustrated in Figure 110A show that treatment with TGFβ for 5 hr induces β-catenin protein expression in MDA-MB-231 cells and this response was decreased by cotreatment with the p38 inhibitor SB202190. TGFβ also induced expression of several EMT marker proteins including Slug, Snail, ZEB-1, N-cadherin, and vimentin; cotreatment with SB202190 inhibited these responses and this was accompanied by increased expression of the epithelial marker ZO-1. Surprisingly cotreatment of TGFβ with LMB or the C-DIM/NR4A1 antagonists also inhibited the β-catenin-regulated protein markers of EMT (Slug, Snail, ZEB-1, N-cadherin, but not vimentin) however, the induced levels of β-catenin were not decreased. As a positive control we also observed that TGFβ-induced Slug and Snail mRNA levels (Fig. 110B). Transfection of MDA-MB-231 cells with p38α (CA), p38α (KD), or p38α (DN) alone or in combination with TGFβ demonstrate that p38α (CA) but not p38α (KD) or p38α (DN) also induce β-catenin and downstream EMT marker proteins (Fig. 110C). TGFβ does not enhance the effects of p38α (CA) and the failure of p38α (KD) or p38α (DN) to induce β-catenin and downstream gene products was not affected by cotreatment with TGFβ.

The role of p38α (CA) in activating β-catenin and downstream EMT gene products was also confirmed in MDA-MB-231 cells treated with TGFβ coupled with knockdown of β-catenin, p38 and p38 upstream kinases TAK1, MKK3 and MKK6. The TGFβ-induced responses were inhibited by decreasing β-catenin,

p38, and upstream genes further confirming the role for p38 in TGFβ-induced EMT. LMB and the C-DIM/NR4A1 antagonists also inhibited induction of the EMT genes by treatment of MDA-MB-231 cells with TGFβ for 5 hr; however, these inhibitors did not affect β-catenin expression (Fig.110A). Results in Figure 7C show that TGFβ induces nuclear β-catenin and SB202190 inhibits thia response; however, cotreatment with LMB results in both nuclear and cytosolic β-catenin whereas β-catenin is primarily cytosolic after cotreatment with TGFβ plus the C-DIM/NR4A1 antagonists. Transfection of p38α also induced expression of β-catenin and EMT markers, where C-DIM/NR4A1 antagonists, LMB, and SB202190 inhbited β-catenin nuclear localization (Fig. 110D) and inhibited the induction of EMT markers (Fig. 110E).

The induction of β-catenin was abrogated by SB202190 (Fig. 110D and 110E), indicating the necessity of p38 in induction of β-catenin and this was also observed when p38 upstream kinases were knocked down (Fig. 110F). The partial nuclear export of β-catenin observed in cells cotreated with TGFβ plus LMB (an exportin-1 inhibitor) may be due, in part, to an equilibrium between nuclear and cytosolic β-catenin and this is currently being investigated. In cells transfected with p38α (CA), p38α (KD), and p38α (DN) alone or in combination with TGFβ (Fig. 110G) the induced β-catenin (by p38α(CA) +/- TGFβ) was nuclear and consistent with activation of EMT (Fig. 7H) however in the other treatment groups β-catenin expression in the nucleus or cytosol was minimal to non-detectable. A possible reason for the difference in β-catenin expression observed

in Figures 100E and 100F may be partially due to the duration of the treatment (5 and 12 hr respectively) and this will be further investigated.

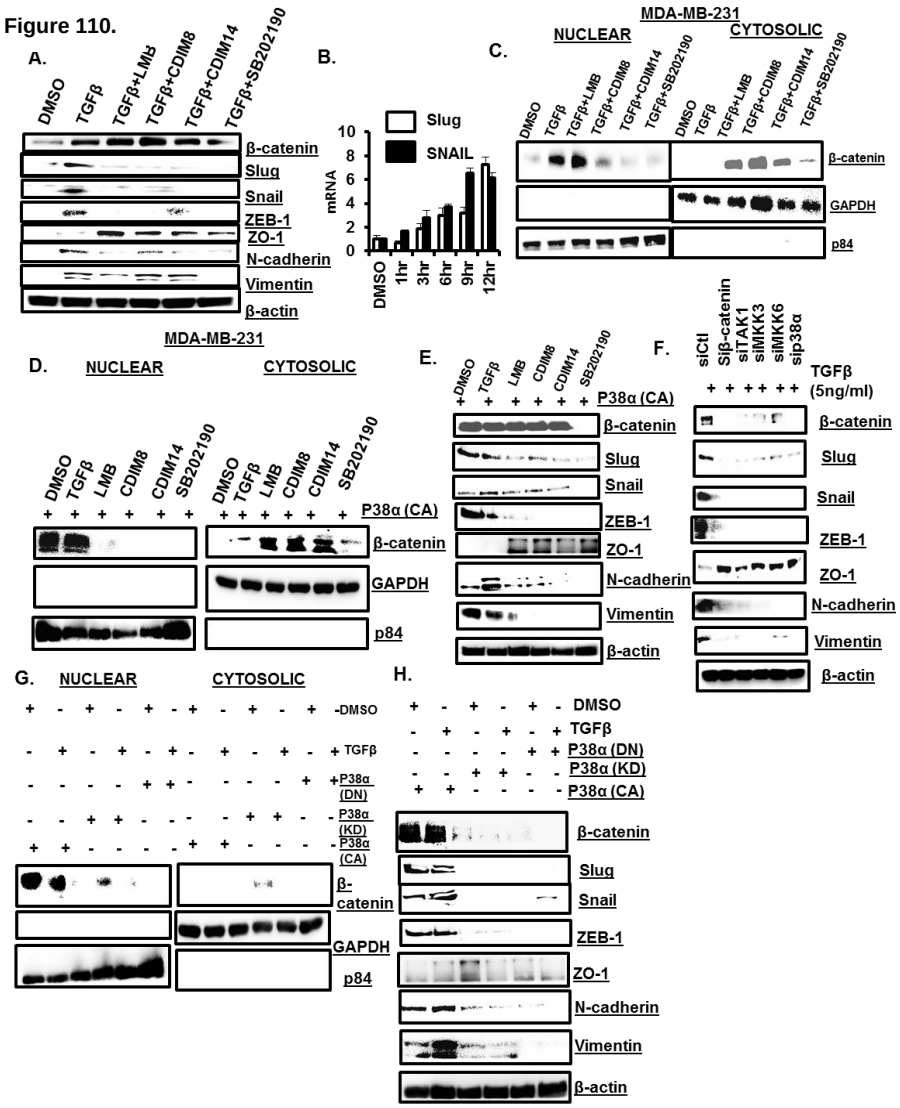

Figure 110: NR4A1 and p38α are required for TGF-β-induced EMT. TGF-β induced Slug and Snail mRNA expression (B) and EMT marker protein expression which was abrogated by C-DIM/NR4A1 anatgonists, LMB, and SB202190 (A). C-DIMs did not reduce β-catenin protein expression but induced its cytosolic sequestration (C). p38α(CA) also induced β-catenin expression (D) and EMT protein expression (E) (which was inhibited by LMB, C-DIM/NR4A1 anatgonists, and SB202190) whereas p38α(KD) and p38α(DN) did not induce expression (G) or EMT protein expression (H) and this was not rescued by TGFβ. Knockdown of kinases upstream of p38α also abrogated induction of EMT protein expression (F).

We also used immunostaining and microscopy to investigate treatment-related cytosolic and nuclear localization of β-catenin. Results demonstrate that p38CA and TGFβ treatment induced nuclear localization (Figure 111A) and β-catenin was sequestered in the cytosol when pretreated with SB202190, leptomycin B, C-DIM 8, and C-DIM 14 (Figure 111A). Both p38(KD) and p38(DN) alone resulted in β-catenin cytosolic sequestration and supplementation with TGFβ did not rescue this effect (Fig. 111B), corroborating results obtained from Figures 110B, 110C and 110F.

Figure 111.

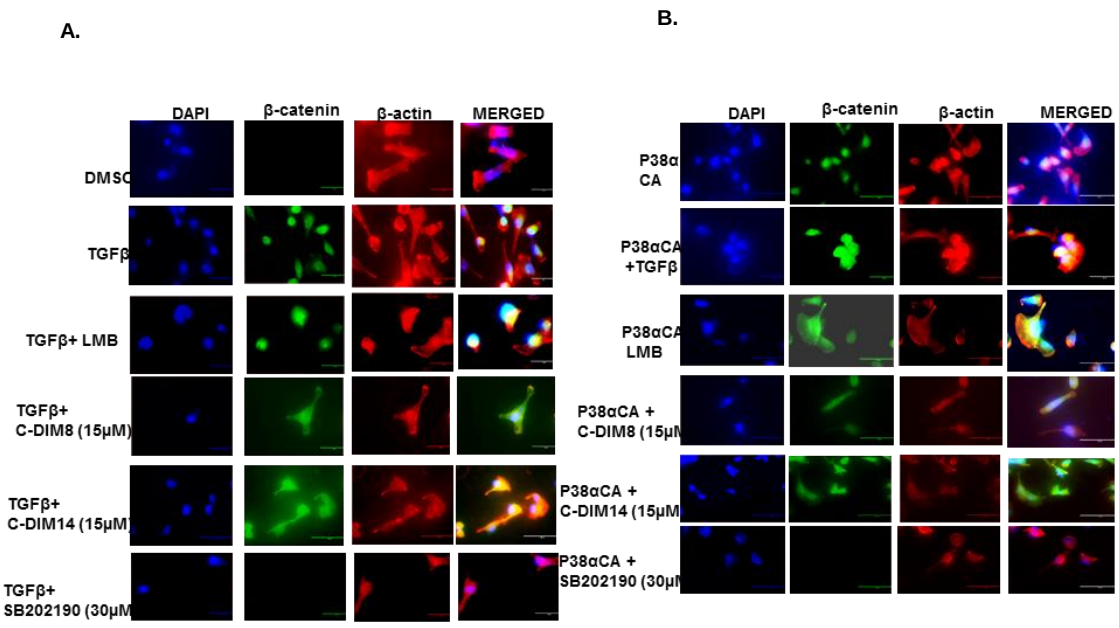

Figure 111. Localization of β-catenin by TGF-β and C-DIMs. Transfection of p38α (CA) induces β-catenin nuclear localization (A) and cotreatment with LMB and C-DIM/NR4A1 antagonists promoted cytosolic sequestration of β-catenin whereas SB202190 abrogated β-catenin expression. Transfection of p38α (KD) or p38α (DN) repressed expression of β-catenin (B) and TGFβ did not rescue this phenotype.

NR4A1 plays a role in regulation of TGFβ-induced β-catenin expression

Results in Figure 7C show that TGFβ-induced β-catenin was primarily nuclear but after cotreatment with NR4A1 antagonists for 5 hr, β-catenin became cytosolic and levels were decreased, suggesting that NR4A1 may play a role in regulating β-catenin expression. This was confirmed in RNAi studies where knockdown of NR4A1 resulted in low to non-detectable levels of TGFβ-induced β-catenin in whole cell lysates (Fig. 112A). In contrast, TGFβ or transfection with p38(CA) induced expression of β-catenin which persisted for 24 hr and remained in the nucleus (Figs. 112B and 112C). In a parallel study after treatment with the NR4A1 antagonist CDIM8, TGFβ- and p38(CA)-induced nuclear β-catenin was exported to the cytosol (Figs. 112D and 112E) as observed in Figure 7C (5 hr treatment with CDIM8); however, after treatment with CDIM8 for 24 hr, the levels of β-catenin were non-detectable. Since cytosolic β-catenin is subject to proteasome-dependent degradation, we repeated the studies outlined in Figures 112D and 112E but in the absence or presence of the proteasome inhibitor MG132.

The results show that MG132 blocked the loss of TGFβ-induced β-catenin in MDA-MB-231 cells (Figs. 112F and 112G). The mechanism of NR4A1 regulation of β-catenin is currently being investigated; however, our results demonstrate that NR4A1 antagonists that inhibit TGFβ-induced nuclear export of NR4A1 induce nuclear export of β-catenin, demonstrating that NR4A1 antagonists

target TGFβ-induced migration and β-catenin-mediated EMT in breast cancer cells through regulating nuclear export of NR4A1.

Figure 112.

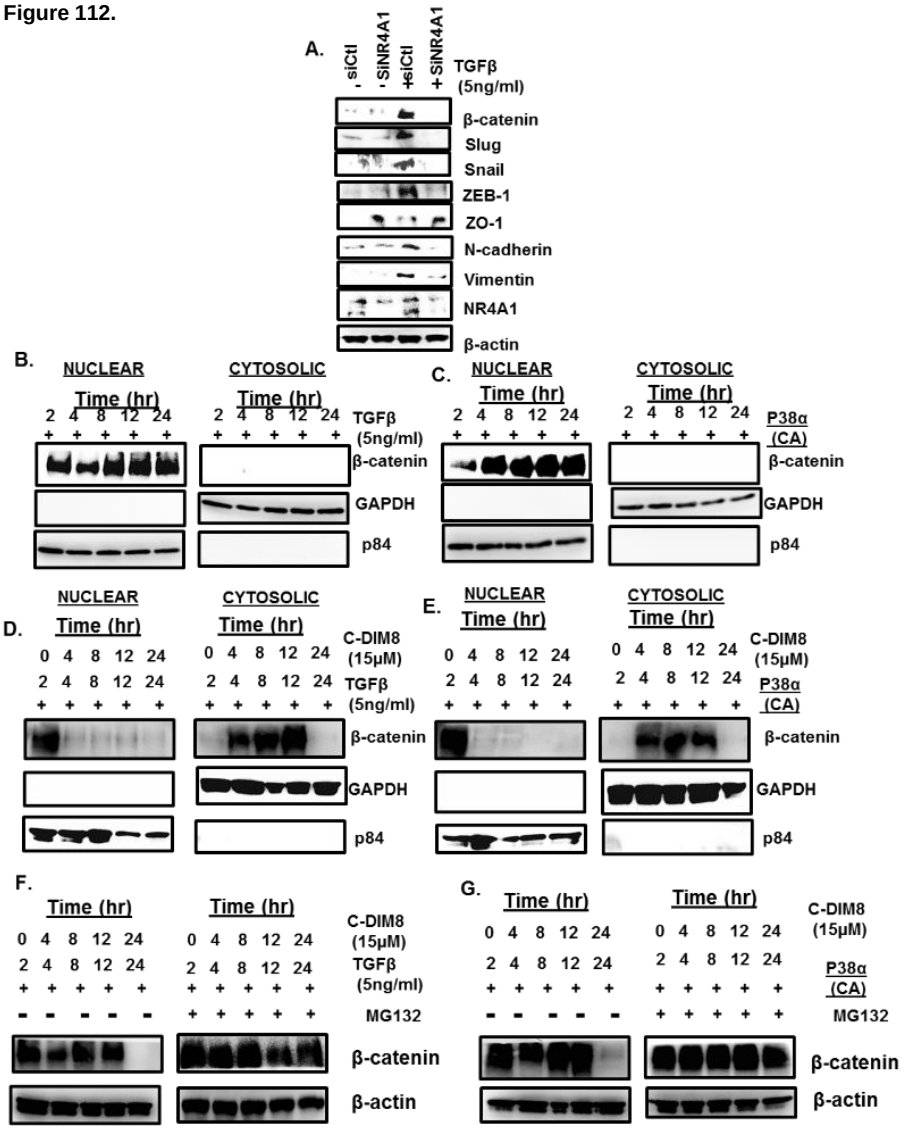

Figure 112. C-DIMs induce proteasome dependent degradation of β-catenin by inducing nuclear export. Knockdown of NR4A1 inhibits TGFβ-induced EMT (A). TGFβ (B) and p38α(CA) (C) both induce nuclear β-catenin over 24 hr timepoint. C-DIM 8 induces cytosolic sequestration of β-catenin at 4 hr in presence of both TGFβ (D) and p38α(CA) (E) which persists for up to 24 hr, leading to degradation of β-catenin. β-catenin expression is restored in the presence of proteasome inhibitor MG132 for both TGFβ (F) p38α(CA) (G).

Role of p38 and β-catenin in regulation of NR4A1 expression and subcellular localization

TGFβ and p38 induce both β-catenin and NR4A1, and NR4A1 regulates β-catenin-mediated expression of EMT genes by maintaining β-catenin in the nucleus (Fig. 110C). Figure 113A illustrates that NR4A1 is nuclear in MDA-MB-231 cells and knockdown of β-catenin, p38 and upstream kinases (TAK1, MKK3 and MKK6) decreased nuclear expression of NR4A1, cytosolic NR4A1 levels were minimal and β-catenin was non-detectable. TGFβ induced NR4A1 and its export to the cytosol and this was accompanied by induction of nuclear β-catenin (Fig. 113B), and knockdown of β-catenin, p38 decreased expression of both NR4A1 and β-catenin and decreased expression of p38α and upstream kinases also blocked nuclear export of NR4A1 as observed in Figures 113A and 113B. Results summarized in Figure 113C show that TGFβ-induced NR4A1 expression is blocked by knockdown of β-catenin, p38 and upstream kinases and using the same treatment protocol TGFβ-induced migration in MDA-MB-231 was also inhibited.

Figure 113.

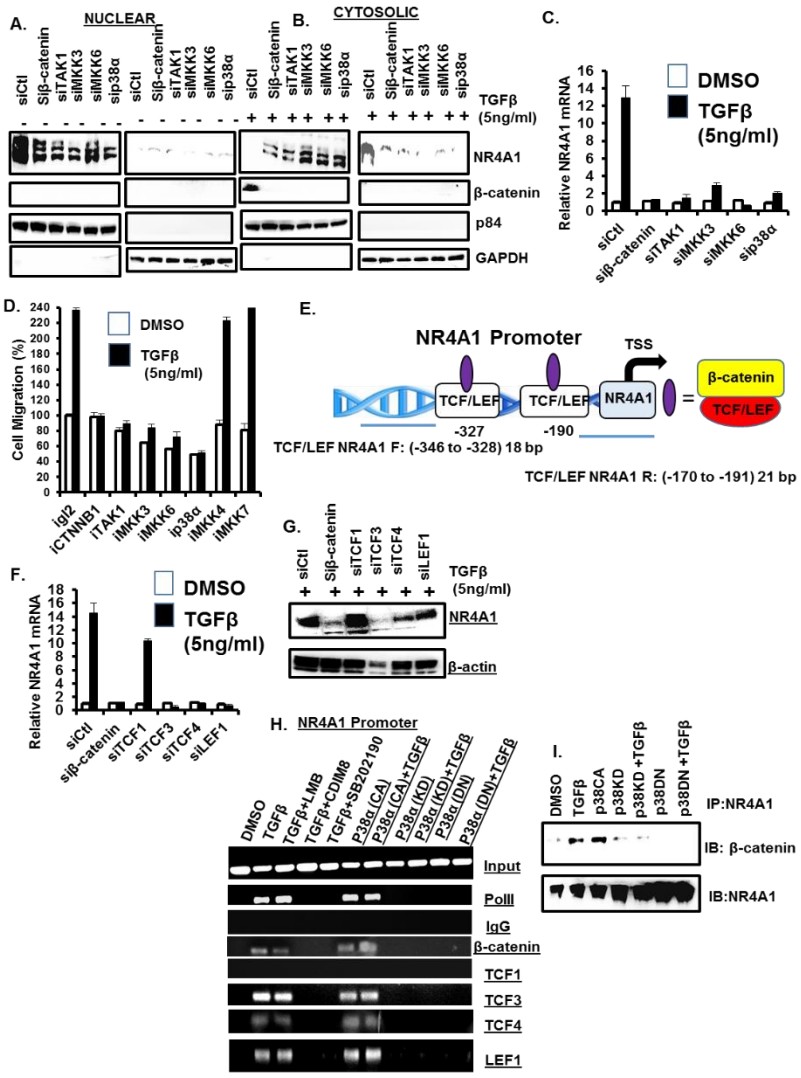

Figure 113. β-catenin and TCF factors induce NR4A1. Knockdown of kinases of upstream of p38α inhibits NR4A1 nuclear export and β-catenin expression (A,B), induction of NR4A1 mRNA (C), and cell migration (D) (where upstream kinases of c-JNK MKK4 and MKK7 did not). Schematic of the NR4A1 promoter with two identified consensus TCF/LEF response elements (TRE) (E), and β-catenin,TCF3, TCF4, and LEF1 associate at these regions However TCF1 was not detected. Knockdown of β-catenin, TCF3, TCF4, and LEF1 (but not TCF1) abrogated TGFβ-induced NR4A1 mRNA (F) and protein expression (G).

β-Catenin is a nuclear transcription factor that regulates gene expression through interactions with DNA-bound TCF/LEF sites and the NR4A1 promoter contains two of these *cis*-elements at -346 to-328 and -191 to -170 (Fig. 113E).

The potential role of β-catenin/TCF/LEF in regulating β-NR4A1 expression was investigated by RNAi and knockdown of β-catenin, TCF3 (siTCF3), TCF4 (siTCF4) and LEF1 (siLEF1) but not TCF1 (siTCF1) decreased TGFβ-induced NR4A1 mRNA (Fig. 113F) and protein (Fig. 113G) levels. Further confirmation that β-catenin regulates NR4A1 gene expression was investigated in a ChIP assay on the NR4A1 promoter which showed that treatment with TGFβ, TGFβ+LMB, p38(CA), and p38(CA)+TGFβ induced pol II and enhanced TCF3, TCF4 and LEF1 binding to the region of the NR4A1 promoter containing the TCF/LEF motifs. In contrast, these promoter interactions were not observed in cells treated/transfected with p38(KD) and p38(DN) alone or in combination with TGFβ or with TGFβ plus the NR4A1 antagonists CDIM8 and CDIM14 (Fig. 113H). These data confirm that β-catenin regulates TGFβ-induced NR4A1 expression (Figs. 113F and 113D), by acting as a nuclear cofactor along with TCF3, TCF4, and LEF1. Figure 114 is a schematic representing the TGFβ/p38α/NR4A1 inducible cell migration and EMT pathway emerging from our studies. TGFβ-inducible migration is dependent on NR4A1 nuclear export and interactions with the Arkadia, Axin2, RNF12 E3 ubiquitin ligase complex that targets SMAD7. This process is NR4A1 and also p38-dependent and can be inhibited by SB202190 and C-DIM/NR4A1 antagonists. TGFβ-induced p38 is also critical for induction of β-catenin and EMT genes and for β-catenin-NR4A1 mutual coregulation where β-catenin acts as a trans-acting transcription factor and NR4A1 and its antagonists regulate β-catenin nuclear export and degradation.

Figure 114.

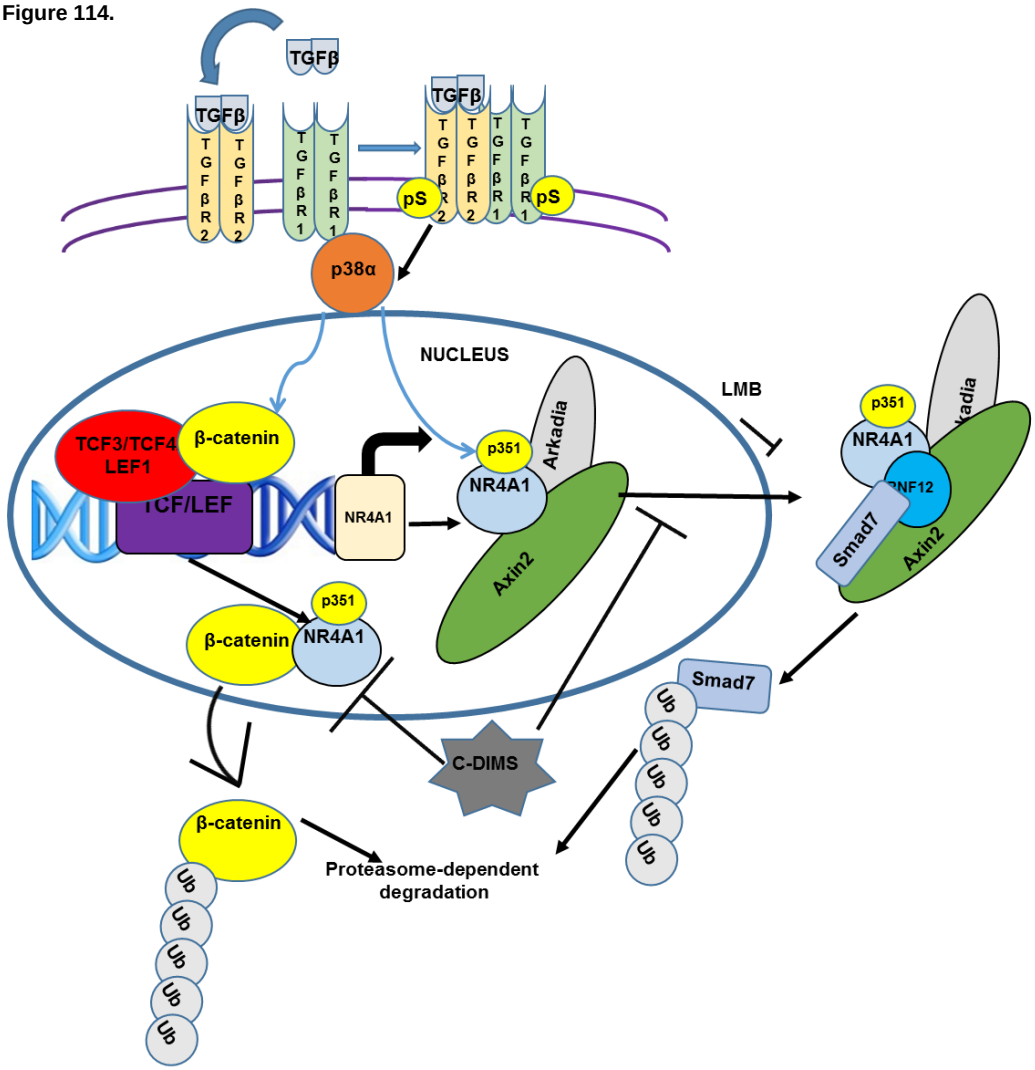

Figure 114. The TGFβ/p38α/NR4A1 pathway. Schematic representing the TGFβp38α/NR4A1 inducible cell migration and EMT pathway.

Discussion

NR4A1 is a member of the NR4A orphan receptor sub-family and although NR4A1 plays a role in multiple physiological and pathophysiological processes, the endogenous ligand for this receptor has not been identified (891,892). Several studies have now characterized ligands that bind NR4A1 (806,910,941,942,1107) and research in this laboratory has investigated the NR4A1 binding of several C-DIM analogs to the receptor (806). NR4A1 is pro-oncogenic in most solid tumors, and both CDIM8 and CDIM14 act as receptor antagonists and inhibit nuclear NR4A1-dependent gene expression, resulting in decreased tumor growth, survival and migration/invasion of breast and other cancer cells (788,803-806,812,904,903). Zhou and coworkers demonstrated that NR4A1 plays a key role in cytokine and TGFβ-induced invasion and EMT in breast cancer by interacting with the Axin2, RNF12 and Arkadia E3 ubiquitin ligase complex which degrades SMAD7 (822). Studies in this laboratory demonstrated that NR4A1-regulation of β1-integrin gene expression is primarily responsible for migration of MDA-MB-231 cells. TGFβ-induced MDA-MB-231 cell migration is also NR4A1-dependent as previously reported (822); however, this is dependent on nuclear export of NR4A1 which is inhibited by the NR4A1 ligand CDIM8 and CDIM14 (903). Thus, the C-DIM/NR4A1 antagonists effectively block TGFβ-induced migration of breast cancer cells, and this study is focused on delineating the mechanisms of TGFβ-NR4A1 interactions.

TGFβ induces multiple kinases including p38 (1097-1099, 1108-1115) and based on initial kinase inhibitor studies in 3 triple negative breast cancer cells (H5587T, MDA-MB-231 and SUM159), we identified p38 as the principle kinase required for nuclear export of NR4A1. Moreover, p38(CA) but not p38(KD) or p38(DN) overexpression induced nuclear export of NR4A1 and subsequent MDA-MB-231 cell migration but both TGFβ- and p38-induced migration were inhibited by C-DIM/NR4A1 antagonists and LMB (Figs.105 A-F). Interestingly, we also observed in these initial experiments that TGFβ and p38(CA) alone or in combination induced the same magnitude of cell migration and other responses, and the lack of activity observed for p38(DN) and p38(KD) was not rescued by cotreatment with TGFβ for any response examined in our studies.

Immunoprecipitation studies with NR4A1 antibodies confirmed that full length NR4A1 interacted with Axin2, Arkadia, RNF12 and SMAD7, and the C-terminal (Axin2, Arkadia and RNF12) and N-terminal (Axin2 and SMAD7) domains of NR4A1 differentially interacted with members of this complex (Fig. 107). Although the functional E3 ubiquitin ligase complex containing NR4A1 is responsible for degradation of SMAD7 (a cytosolic protein) (822), we observed that like NR4A1, Arkadia was also a nuclear protein that underwent TGFβ/p38-dependent nuclear export (Fig. 108G). Moreover, the NR4A1 antagonists CDIM8 and CDIM14 that inhibited TGFβ-/p38-mediated nuclear export of NR4A1 also inhibited nuclear export of Arkadia. NR4A1 forms protein-protein complexes with

other nuclear factors such as p53 and LKB1 and treatment with receptor ligand induces dissociation from the receptor (941,1116), whereas NR4A1-Arkadia nuclear interactions remained intact in the presence or absence of ligand (Fig. 108G). The ultimate target of the E3 ubiquitin ligase complex is SMAD7 (822), and we also observed the TGFβ-/p38-induced proteasome-dependent SMAD7 ubiquitination and degradation. Moreover, inhibitors of NR4A1 (and Arkadia) nuclear export or knockdown of one or more members of this complex also decreased SMAD7 ubiquitination, and these observations are consistent with studies showing the importance of this complex in SMAD7 degradation (822).

Previous studies show that TGFβ induces cancer cell migration and EMT, and activation of the latter pathway may also involve increased expression of β-catenin (1117-1119). In addition, it has also been reported that β-catenin not only interacts with NR4A1 but there is evidence for mutual functional responses and inter-protein regulatory pathways (1120-1125). Knockdown or overexpression of NR4A1 in colon cancer cells decreases or increases β-catenin expression, respectively, and knockdown or overexpression of β-catenin decreases or increases NR4A1 expression (1120-1122). Hypoxia also enhances β-catenin/NR4A1-mediated invasion of colon cancer cells; however, the mechanism of this response and the intracellular location of NR4A1 are unclear, and the role of NR4A1 is independent of DNA binding and transactivation (1122). The role of NR4A1 in TGFβ-induced expression of β-catenin and downstream EMT genes in

MDA-MB-231 cell is unique. Both TGFβ and p38(CA) induced expression of β-catenin and downstream genes in MDA-MB-231 cells and this response was inhibited not only by p38(DN), p38(KD) and knockdown of kinases upstream from p38, but also by NR4A1 antagonists CDIM8 and CDIM14 (Figs. 110 and 111). Treatment with TGFβ resulted in induced β-catenin expression in the nucleus and nuclear export of NR4A1, and the effects of NR4A1 antagonists (5 hr treatment) reversed the subcellular location of both proteins, suggesting nuclear (antagonist) ligand-bound NR4A1 either directly or indirectly enhanced nuclear export of β-catenin. Moreover, CDIM8- and CDIM14-induced nuclear export of β-catenin resulted in proteasome-dependent degradation of this protein within 24 hr after initial treatment (Fig. 112). Knockdown of NR4A1 by RNA also decreased β-catenin expression (Fig. 112A), suggesting that the loss or inactivation of NR4A1-regulated genes/pathways contributed to nuclear export of β-catenin and its subsequent degradation, and this is currently being investigated.

β-Catenin overexpression or induction in colon cancer cells induces NR4A1 expression and this is linked to activation of AP1 and subsequent interactions with AP1 *cis*-elements in the proximal region (-200 to -2) of the NR4A1 gene promoter (1121). In contrast, TGFβ-induced β-catenin-mediated induction of NR4A1 was due to nuclear β-catenin interactions with TCF3/TCF4 (but not TCF1) and LEF *cis*-element (-346 to -328 and -190 to -170) in the proximal region of the NR4A1 gene promoter (Figs. 113 and 114). These interactions were TGFβ-

dependent and illustrate a unique crosstalk between NR4A1 and β-catenin which are critical elements in late stage TGFβ-induced breast cancer cell migration/invasion. Our results demonstrate that these TGFβ-induced responses are also dependent on induction of p38 and genomic regulation of NR4A1 by β-catenin/TCF/LEF complexes. In contrast, TGFβ/p38-dependent induction of β-catenin results in nuclear accumulation of β-catenin and activation of downstream EMT genes. The precise role for NR4A1 in mediating TGFβ/p38-dependent induction of β-catenin is unknown; however, NR4A1 antagonists that retain NR4A1 in the nucleus also induce β-catenin nuclear export and degradation demonstrating a novel NR4A1 ligand-dependent approach for inhibiting EMT in breast cancer cells.

CHAPTER X

SUMMARY AND CONCLUSIONS

NR4A1 is overexpressed in transformed tissue as compared to its normal counterpart in the majority of solid tumors. We have developed structurally-diverse C-DIMs that bind to NR4A1 and antagonize its ability to serve as a transcription factor for expression of a myriad of oncogenes that are vital for tumorigenesis, evasion of apoptosis, suppression of ROS, migration, and invasion. Specifically, this laboratory has investigated C-DIMs and their molecular mechanisms as anticancer agents in breast, kidney, colon, pancreatic, RMS, and other cancer cell lines Two NR4A1 ligands, 1,1-bis (3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH) (C-DIM 8) and the *p*-carbomethoxy derivate (DIM-C-pPhCO_2Me) (C-DIM 14) inhibited cancer cell proliferation and induced apoptosis. C-DIMs inhibited cancer cell growth by upregulating sestrin 2 (SESN2) which activated AMPKα resulting in downregulation of mechanistic target of rapamycin (mTOR) signaling. These results were observed in both p53 wt. and p53 mutant cell lines, and demonstrated that SESN2 can be induced in a p53 dependent and a p53 independent (ROS-induced) manner. This in it of itself represents a new approach to targeting p53 mutated cancers as this effect was observed in all p53 mutant cell lines used in this dissertation. C-DIMs also induced apoptosis by activating caspases 3, 7, 8, inducing poly ADP-ribose

polymerase (PARP) cleavage, which was corroborated using Annexin V staining. We also investigated two ROS mediators of apoptosis that were identified by RNA-seq analysis in cells after NR4A1 siRNA-mediated knockdown or treatment with NR4A1 antagonist. Knockdown of NR4A1 and C-DIM/NR4A1 antagonists downregulated expression of thioredoxin domain containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1) and ROS was induced as determined by flow cytometry. The endoplasmic reticulum (ER) stress pathway was also activated as indicated by increased expression of ER stress markers: CHOP, XBP-1s, ATF4, p-PERK, ATF6, GRP78, and IRE1. Similar results were obtained for all these pathways using RNA interference (RNAi) and oligonucleotides that target NR4A1 (siNR4A1). These results indicate that C-DIMs are effective NR4A1 antagonists and inhibit NR4A1-dependent oncogenic pathways. Therefore, C-DIMs exhibit promising chemotherapeutic potential NR4A1-overexpressing solid tumors observed.

β1-Integrin as determined by RNA-seq analysis as a downstream target of both siNR4A1 and NR4A1 antagonists. In breast, pancreatic, and colon cancer, β1-Integrin is highly expressed and it is associated with a negative prognosis including disease recurrence, metastasis and decreased overall and disease-free survival. Results of studies *in vitro* and *in vivo* preclinical models show that β1-integrin plays an important role in mammary tumor initiation, progression and metastasis. NR4A1 has been demonstrated to coregulate oncogenic proteins along with p300 and Sp1. We observed that NR4A1 regulates expression of both

β1- and β3-integrins through this transactivational complex and unlike other β1-integrin inhibitors, which induce prometastatic β3-integrin; NR4A1 antagonists inhibit expression of both β1- and β3-integrin demonstrating a novel mechanism-based approach for targeting integrins and integrin-dependent breast cancer metastasis. This transactivational complex was also shown to be necessary for expression of other integrins such as β4-, α5-, and α-6 integrin in Chapter VIII, which can be targeted by ROS inducers like penfluridol. However, this dissertation also demonstrated that not only does Sp1 cooperate in this transactivation complex, but Sp3 and Sp4 on certain oncogenic promoters. Indeed, in RKO, L3.6pL, MiaPaCa2 and Panc1 cells it was Sp3 and Sp4, not Sp1 that was demonstrated to be required for transactivation of β1-integrin. Therefore, this illustrates that Sp proteins have unique roles in tumorigenesis and cancer cell migration, which is cell context specific.

Overexpression of NR4A1 in ER positivie and ER negative breast cancer. A specific subtype, triple negative breast cancer (TNBC) is refractory to most chemotherapeutic regimens and NR4A1 is a prognostic factor for decreased survival and increased metastasis. Recently, metastasis has been linked to NR4A1-dependent regulation of TGFβ signaling, which is a powerful driver of epithelial to mesenchymal transition (EMT), migration/invasion, and progression of metastatic cancer. NR4A1 is involved in TGFβ induced invasion in breast cancer by forming a complex with Axin2, RNF12, and ARKADIA, which catalyze ubiquitination of SMAD7, leading to its proteasome-dependent degradation, and

activation of TGF-β induced cell migration by stabilization of the TGFβR1 receptor. We provide insight into the mechanism of TGFβ mediated cell migration and that this requires nongenomic NR4A1 function. We demonstrate that migration of aggressive SKBR3 and MDA-MB-231 breast cancer cells is constitutive, TGFβ-independent, and dependent on regulation of β1-integrin gene expression by NR4A1 which can be inhibited by the NR4A1 antagonists (DIM-C-pPhOH) and (DIM-C-pPhCO_2Me) analog. The NR4A1 antagonists also inhibited TGFβ-induced migration of MDA-MB-231 cells by blocking nuclear export of NR4A1, which is an essential step in TGFβ-induced cell migration.

We also demonstrate that p38α is necessary and sufficient for TGFβ-mediated migration, NR4A1 nuclear export, and NR4A1 induction by induction of β-catenin in triple negative breast cancer (TNBC) as overexpression of p38CA recapitulated TGFβ-mediated NR4A1 nuclear export and cancer cell migration and was attenuated with NR4A1 antagonists (C-DIMs), leptomycin B, and the p38 inhibitor SB202190. The variants p38KD (kinase-dead) and p38DN (dominant negative) did not recapitulate the effects of p38CA and subsequent TGFβ treatment failed to rescue this phenotype. We also demonstrate that NR4A1 is essential for TGFβ-induced EMT as expression of EMT markers was abrogated in the presence of NR4A1 antagonists and SB202190. Moreover, NR4A1 antagonists promoted cytosolic sequestration of the transcription factor β-catenin and its proteasome-dependent degradation in a time dependent manner. TCF/LEF response elements (TRE) were identified within the NR4A1 promoter

and chromatin immunoprecipitation analysis demonstrated that β-catenin (with TCF-3, TCF-4, and LEF-1) binds these TREs. We also demonstrate that β-catenin is required for TGFβ-dependent NR4A1 induction and is p38α-dependent. These results highlight a novel mechanism of TGFβ/NR4A1-dependent inducible migration and demonstrate therapeutic potential of NR41 in TNBC. Our results show mutual coregulation of NR4A1 and β-catenin play a role in TGFβ-induced TNBC cell migration and EMT and NR4A1 antagonists inhibit this response and demonstrate a novel approach for TNBC chemotherapy for patients overexpressing NR4A1.

Specificity protein (Sp) transcription factor (TF) Sp1 is overexpressed in multiple tumors and is a negative prognostic factor for patient survival. Sp1, Sp3 and Sp4 are highly expressed in cancer cells and in this study, we demonstrate that these TFs individually play a role in cancer cell growth, survival and migration/invasion in SKBR3, MDA-MB-231 breast, 786-0 kidney, L3.6pL, PANC1, Miapaca2 pancreatic, A549 lung and SW480 colon cancer cell lines. Surprisingly, after individual knockdown of Sp1, Sp3, or Sp4, which resulted in inhibition of cell growth and migration and induction of apoptosis, compensation by the other expressed Sp proteins, was not observed. This underscores the necessity of all three Sp proteins in driving tumorigenesis in multiple tumor types. Moreover, tumor growth in athymic nude mice bearing L3.6pL pancreatic cancer cells as xenografts was significantly attenuated in cells were depleted of Sp1, Sp3, and Sp4 in combination or Sp1 alone. There was also a significant difference

between the combinatorially knocked down tumors and the ones that had Sp1 alone, further demonstrating that Sp3 and Sp4 are vital for tumor growth. Ingenuity Pathway Analysis (IPA) of changes in gene expression in Panc1 pancreatic cancer cells after individual knockdown of Sp1, Sp3 and Sp4 demonstrates that these TFs regulate gene sets/pathways that both correlate and inversely correlate with the functional responses observed after knockdown. Causal IPA analysis also predicted that Sp1-, Sp3- and Sp4-regulated genes were associated with the pro-oncogenic activity and that the moajority of these were not mutaually exclusive. These functional and genomic results coupled with overexpression of Sp transcription factors in tumor vs. non-tumor tissues and decreased Sp1 expression with age indicate that Sp1, Sp3 and Sp4 are non-oncogene addiction (NOA) genes and are attractive drug targets for individual and combined cancer chemotherapies.

These SP proteins were also demonstrated to be vital for RMS cell and tumor growth. Both the ERMS and ARMS cell lines were inhibited by panobinostat treatment with downregulated Sp proteins in a non-canonical fashion. This involved the c-myc/miR 17-92/ZBTB/Sp pathway, which is iniated by ROS, which induce epigenetic changes in the c-myc promoter. Therefore, development of innovative chemotherapeutic strategies is imperative and a recent genomic analysis suggested the potential efficacy of reactive oxygen species (ROS)-inducing agents. Here we demonstrate the efficacy of potent histone deacetylase (HDAC) inhibitors, panobinostat and vorinostat, as agents that inhibit tumor

growth *in vivo*, induce apoptosis, and inhibit invasion of RMS cell lines RD and Rh30. These effects are due to ROS-dependent epigenetic repression of c-Myc, which leads to downregulation of miRs 17, 20a, 27a, upregulation of ZBTB 4, 10, 34 and subsequent downregulation of Sp transcription factors. We also demonstrate that this is an ROS-dependent mechanism and treatment with the antioxidant glutathione reversed the effects of the HDAC inhibitor substantially, while histone acetylation was unchanged. These results elucidate a novel mechanism of antineoplastic activity for panobinostat and vorinostat that lies outside of their canonical HDAC inhibitory activity and present a potential new antitumorigenic approach to treat RMS, whether to be used individually or as an adjuvant to an existing chemotherapeutic regimen.

Overall this dissertation has provided substantial evidence that NR4A1 and Sp transcription factors are potent oncogenes, with the former having both genomic and nongenomic oncogenic functions which can be targeted by our C-DIM/NR4A1 antagonists and Sp proteins can be targeted by ROS inducers and phytochemicals. We not only demonstrate the potency both *in vitro* and *in vivo,* but elucidate a mechanism that is physiologically relevant to many aggressive, drug-refractory cancers. Both NR4A1 and Sp proteins are drug targetable and represent a promising class of chemotherapeutic targets for solid tumors and metastasis.

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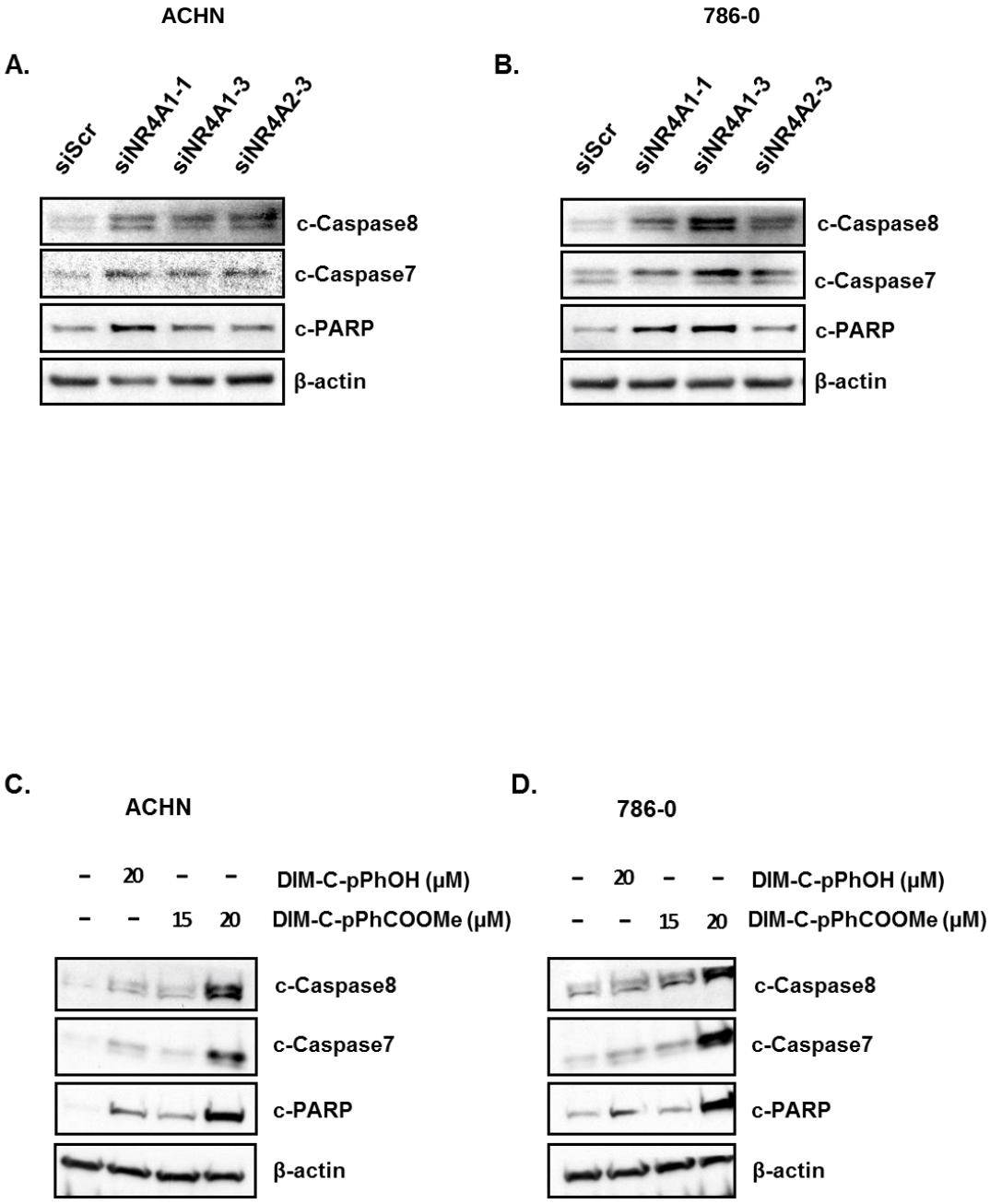
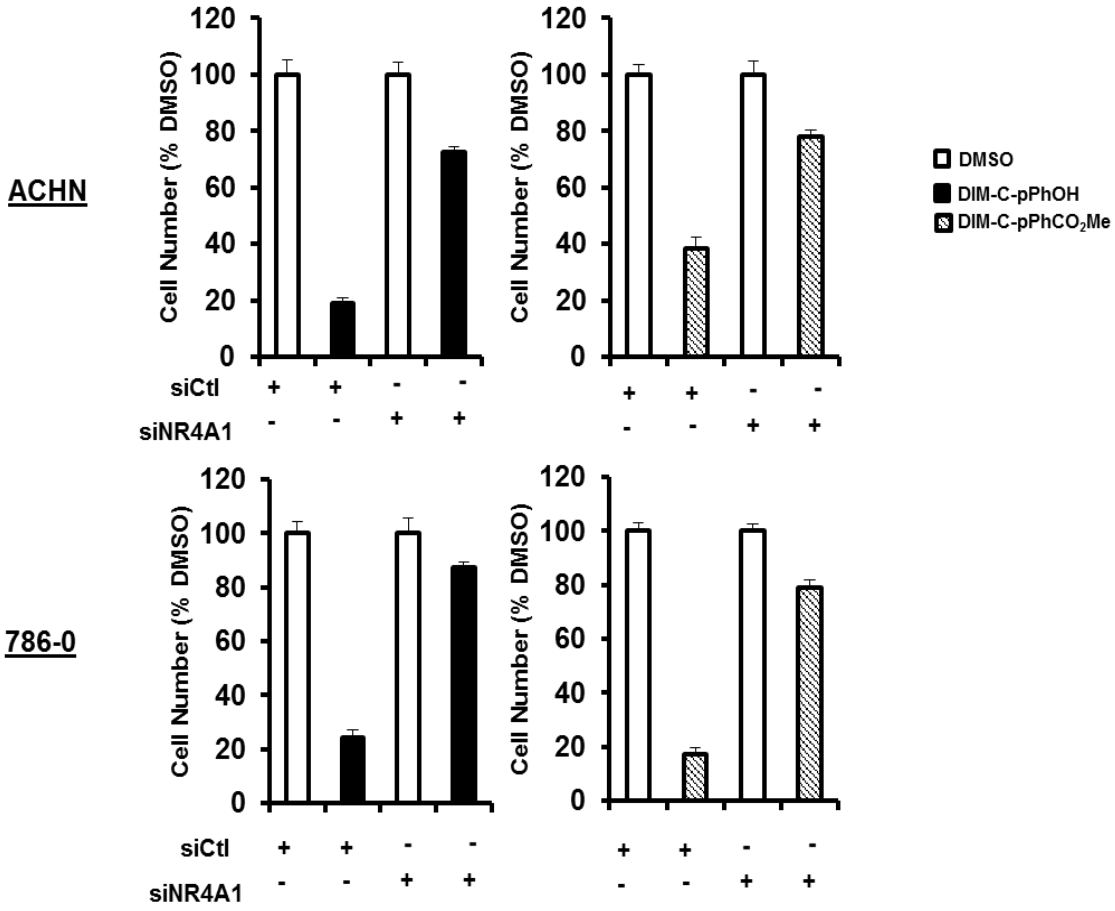
APPENDIX A

Supplemtal Figure A-1



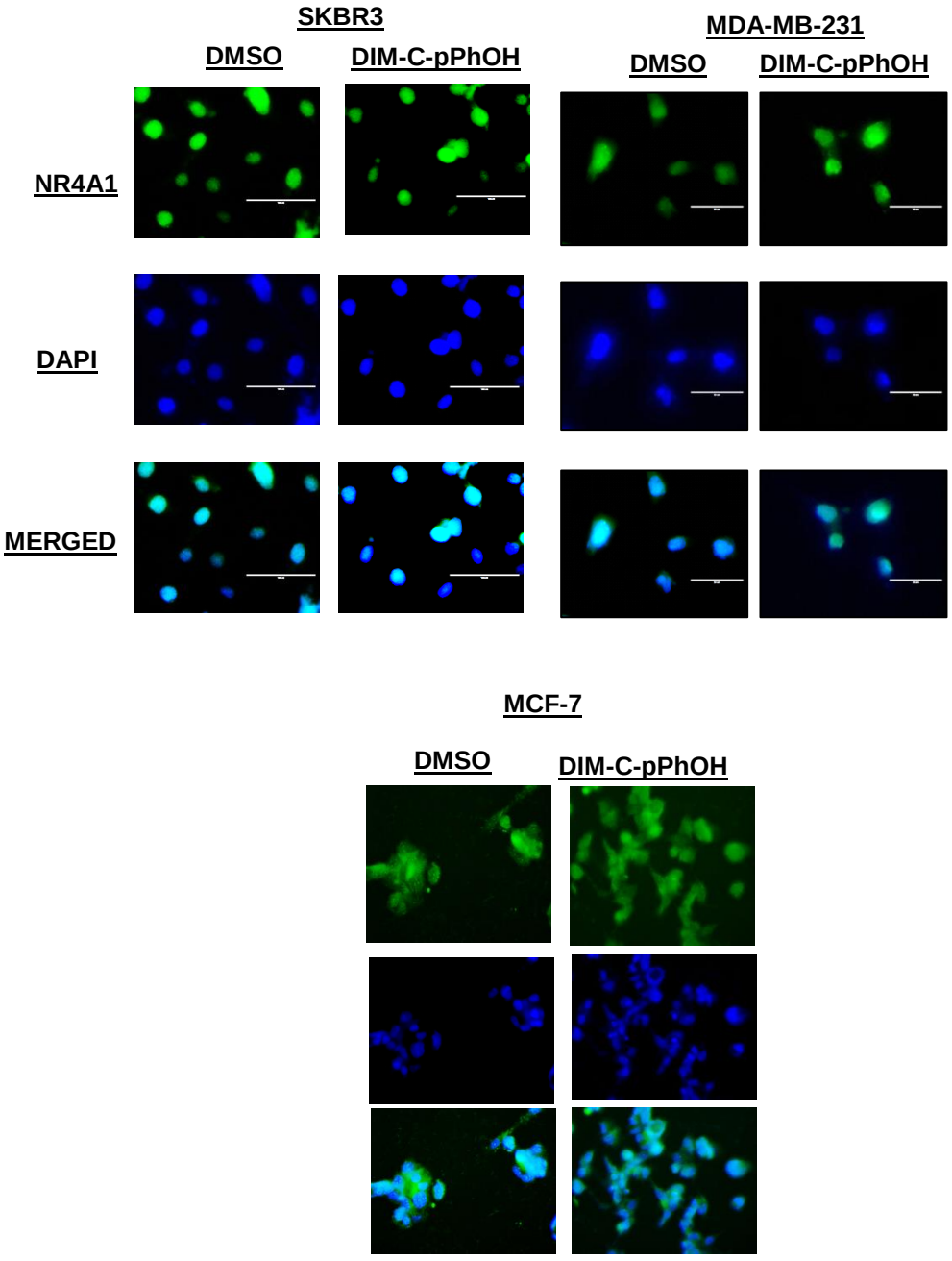
Supplemental Fig A-1. Different oligonucleotides targeting NR4A1 induce apoptosis. Different oligonucleotides targeting NR4A1 induce caspase 8,7 and PARP cleavage in ACHN (A) and 786-0 (B) cancer cells and this is also observed with DIM-C-pPhOH and DIM-C-pPhCOOMe in ACHN (C) and 786-0 (D) cells.

Supplemental Figure A-2.



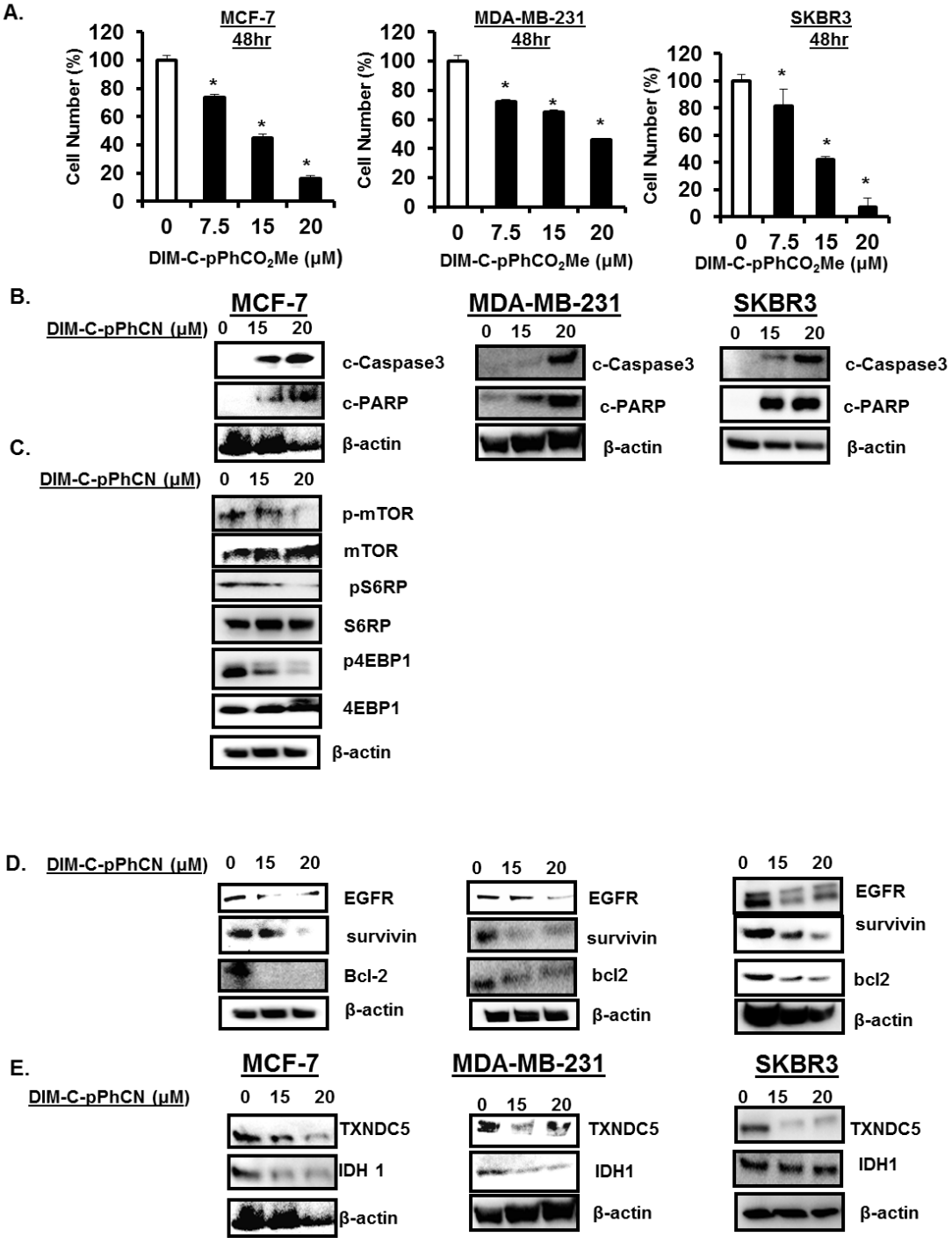
Supplemental Fig A-2. C-DIMs inhibit cell proliferation through inactivation of NR4A1. DIM-C-pPhOH and DIM-C-pPhCO$_2$Me required NR4A1 to inhibit cell proliferation in ACHN and 786-0 cells.

Supplemental Figure A-3.



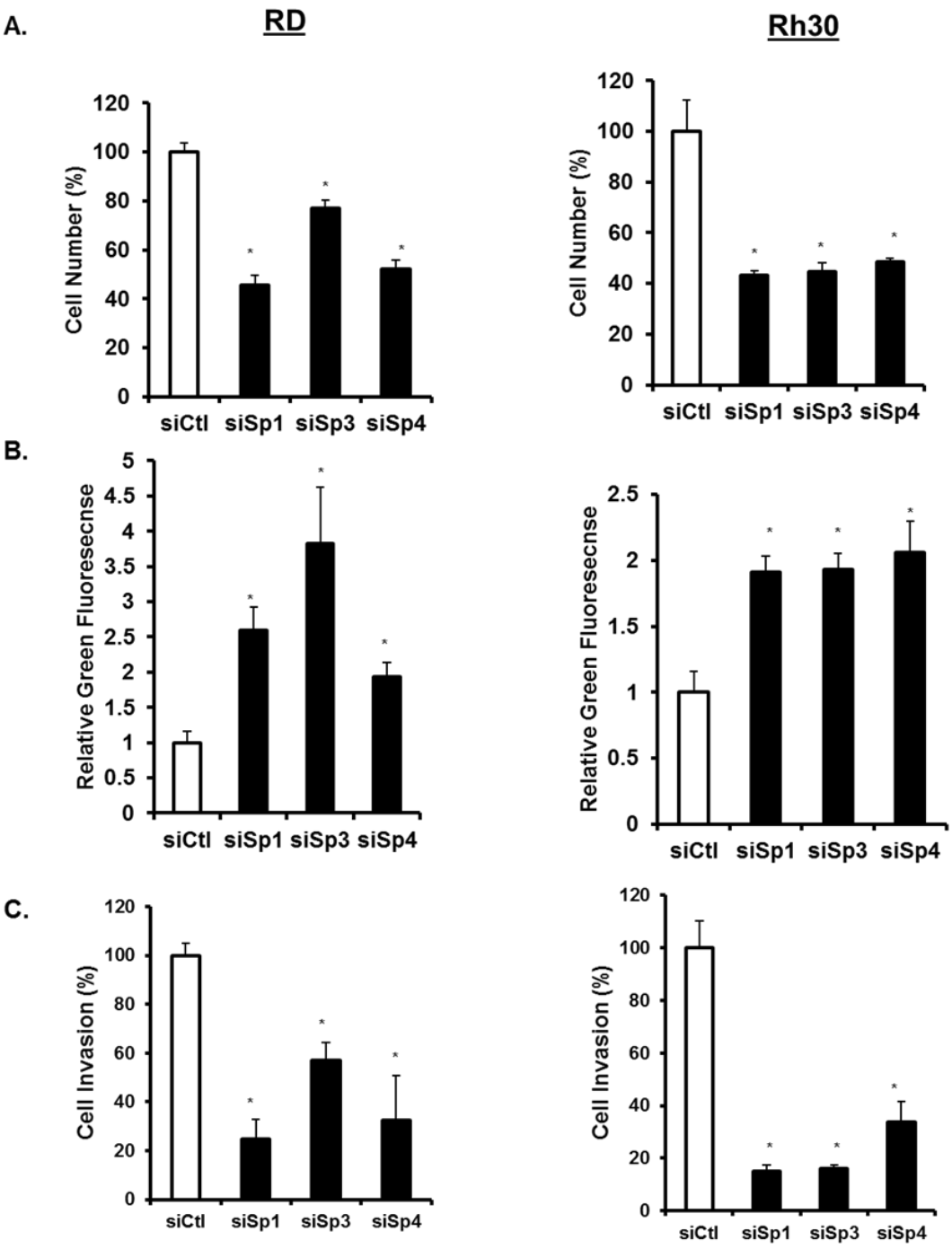
Supplemental Fig A3-: DIM-C-pPhOH inhibits nuclear NR4A1. NR4A1 remains nuclear after treatment with NR4A1 antagonist DIM-C-pPHOH (20 μM)

Supplemental Figure A-4



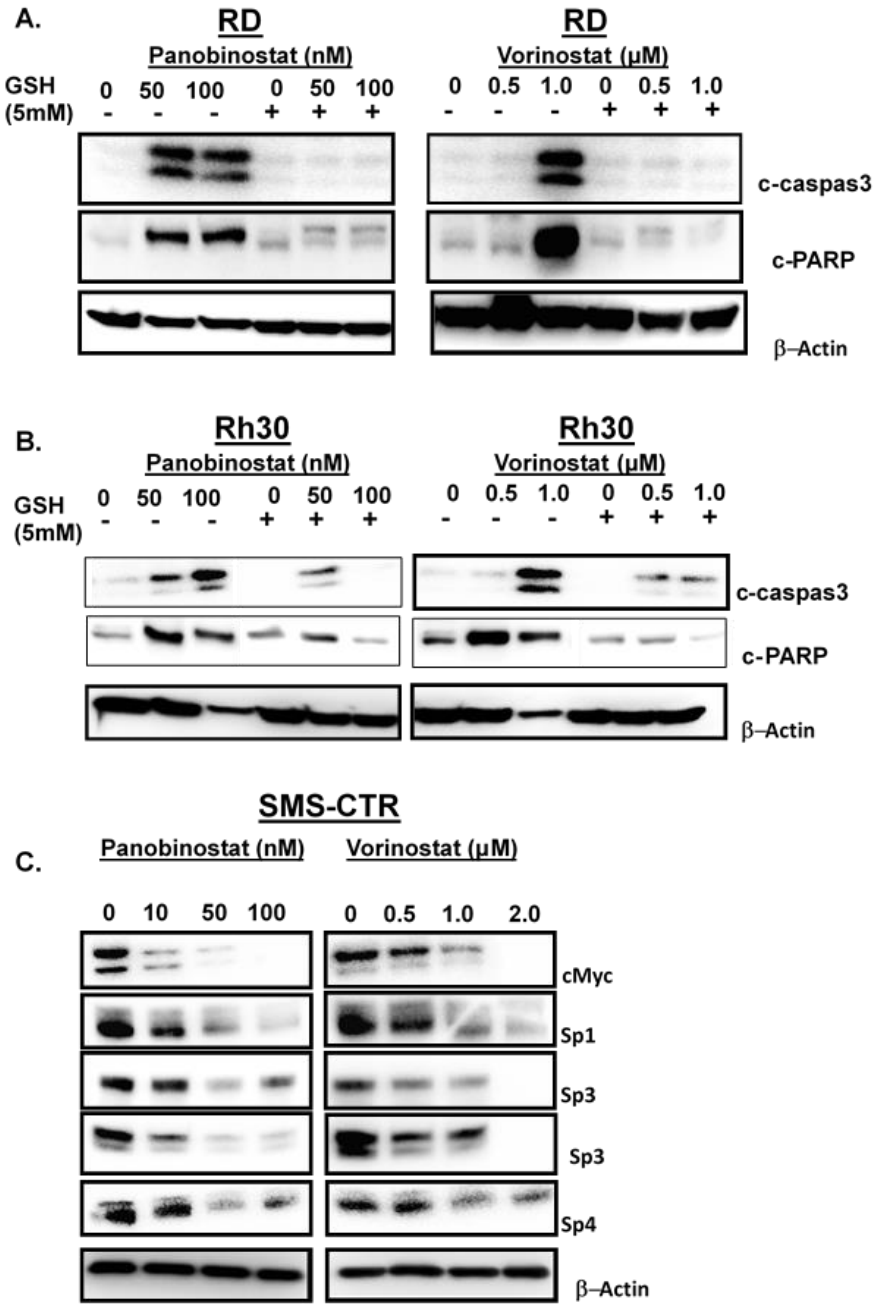
Supplemental Figure A-4. DIM-C-pPhCO$_2$Me inhibits cell proliferation up to 48 hrs and DIM-C-pPhCN has similar effects on breast cancer cells. The C-DIMs DIM-C-pPhOH and DIM-C-pPhCO$_2$Me decrease cell proliferation in MCF-7, MDA-MB-231, and SKBR3 cell lines at 24 and 48 hrs (A). DIM-C-pPhCN induces PARP and caspase 3 cleavage (B), inhibits mTOR signaling (C), downregulates Sp regulated protein expression (D), and downregulates TXNDC% and IDH1 (E).

Supplemental Figure A-5



Supplemental Figure A-5. Sp knockdown inhibits growth, induces apoptosis, and dcreases RMS cell invasion. RD and Rh30 cells were transfected with siCtl, siSp1, siSp3 and siSp4, and effects on cell proliferation (A), apoptosis (B) and invasion (C) were determined by counting cells (Coulter counter), fluorescent detection of Annexin V and Boyden chamber assays, respectively. Results are expressed as means ± SE for 3 replicate determinations and significant ($p<0.05$) effects compared to siCtl (set at 100%) are indicated (*).

Supplemental Figure A-6



Supplemental Figure A-6. HDACi downregulate c-Myc and Sp proteins in SMS-CTR ERMS cells. Panobinostat and vorinostat both induce PARP and caspase 3 cleavage in RD (A) and Rh30 (B) cells which is ameliorated by cotreatment with GSH (A,B). Panobinostat and vorinostat also downregulate c-Myc and Sp proteins in ERMS cell line SMS-CTR (C).

Supplemental Figure A-7

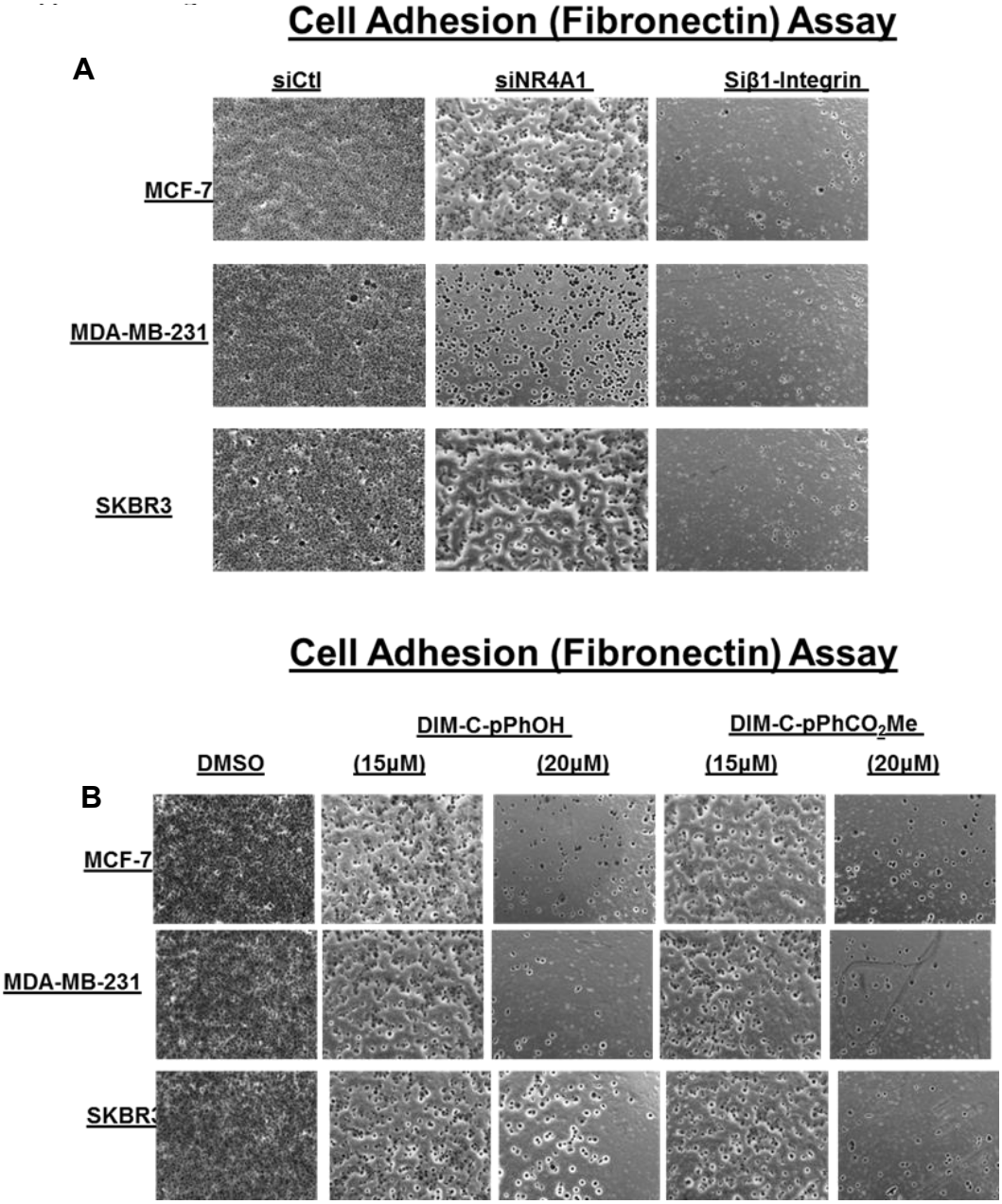


Figure A-7. NR4A1 knockdown and C-DIMs inhibit cell adhesion. Cells were transfected with siNR4A1 or treated with NR4A1 antagonists and fibronectin-induced cell adhesion was determined. (A). Cells were transfected with siNR4A1 or siITGB1 (β1-integrin), treated with DIM-C-pPhOH (20 μM), and cell adhesion was determined(B).

Supplemental Figure A-8

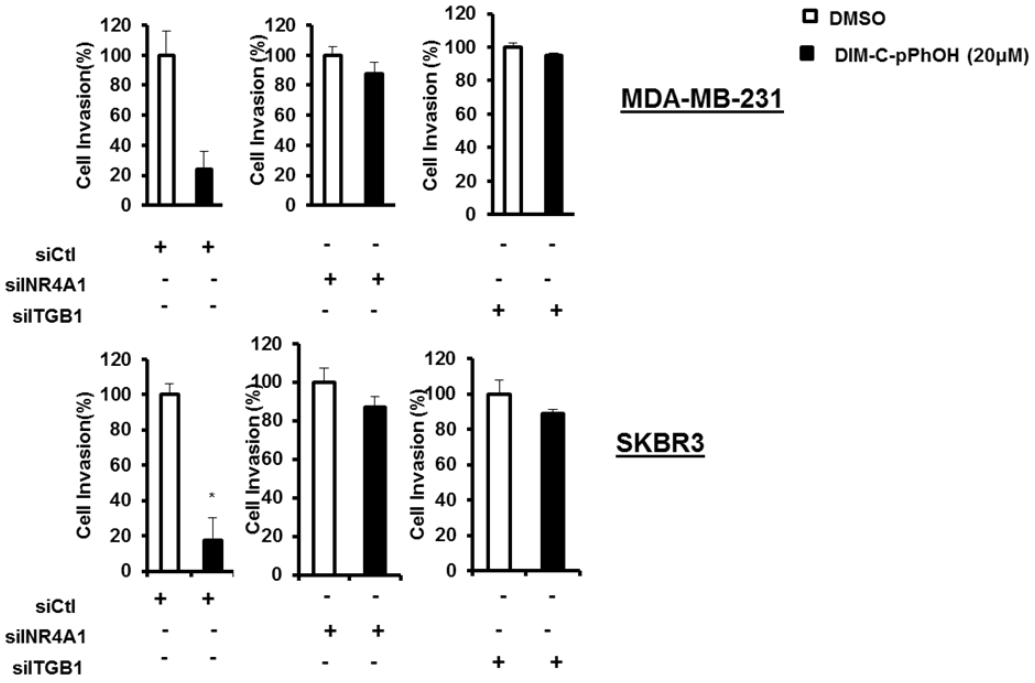


Figure A-8. DIM-C-pPhOH requires NR4A1 and β1-integrint cell migration. Cells were transfected with siNR4A1 or siITGB1 (β1-integrin), treated with DIM-C-pPhOH (20 μM), and cell migration was determined. Results are expressed as means +/- SE (3 replicates) and significantly (*P*<0.05) decreased migration is indicated (*).

Supplemental Figure A-9

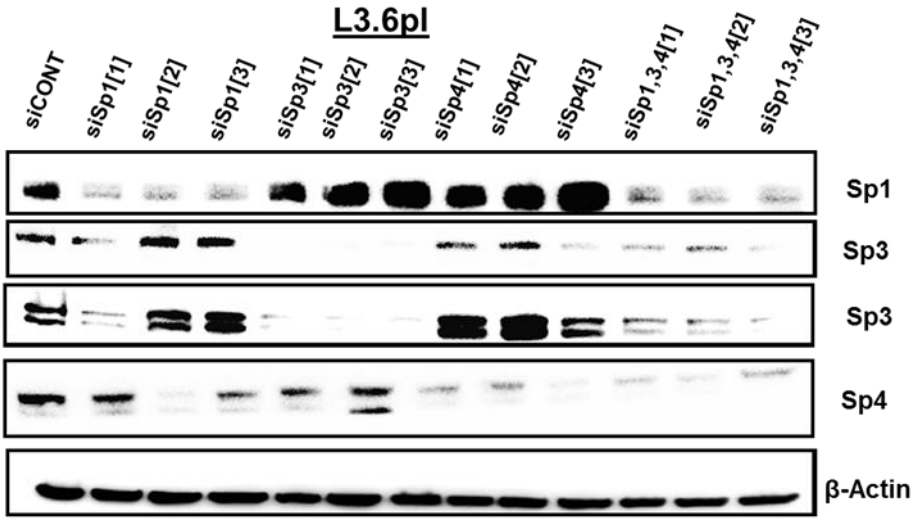
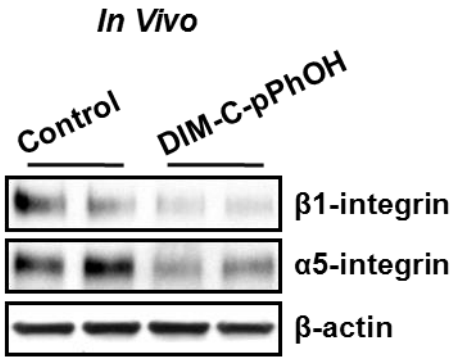


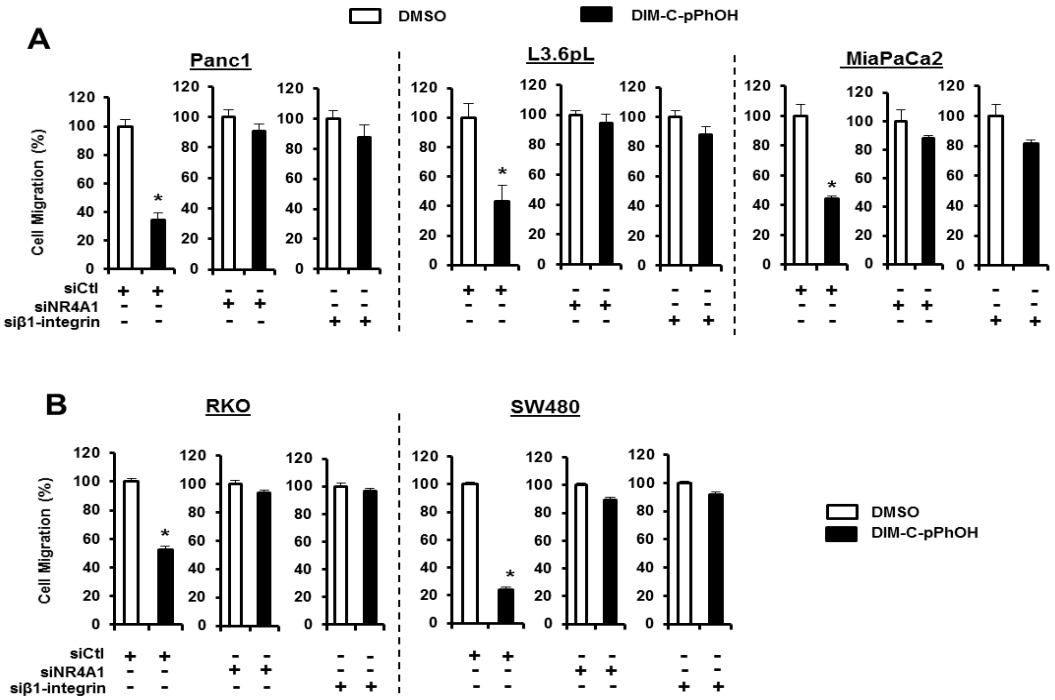
Figure A-9. Effects of different Sp oligonucleotides. Multiple Sp oligonucleotides targeted against Sp1,Sp3, and Sp4 were transfected into L3.6PL cells and whole cell lysates were analyzed by Western blots. Sp1(1), Sp3(1), and Sp4(1) were used in subsequent knockdown experiments.

Supplemental Figure A-10



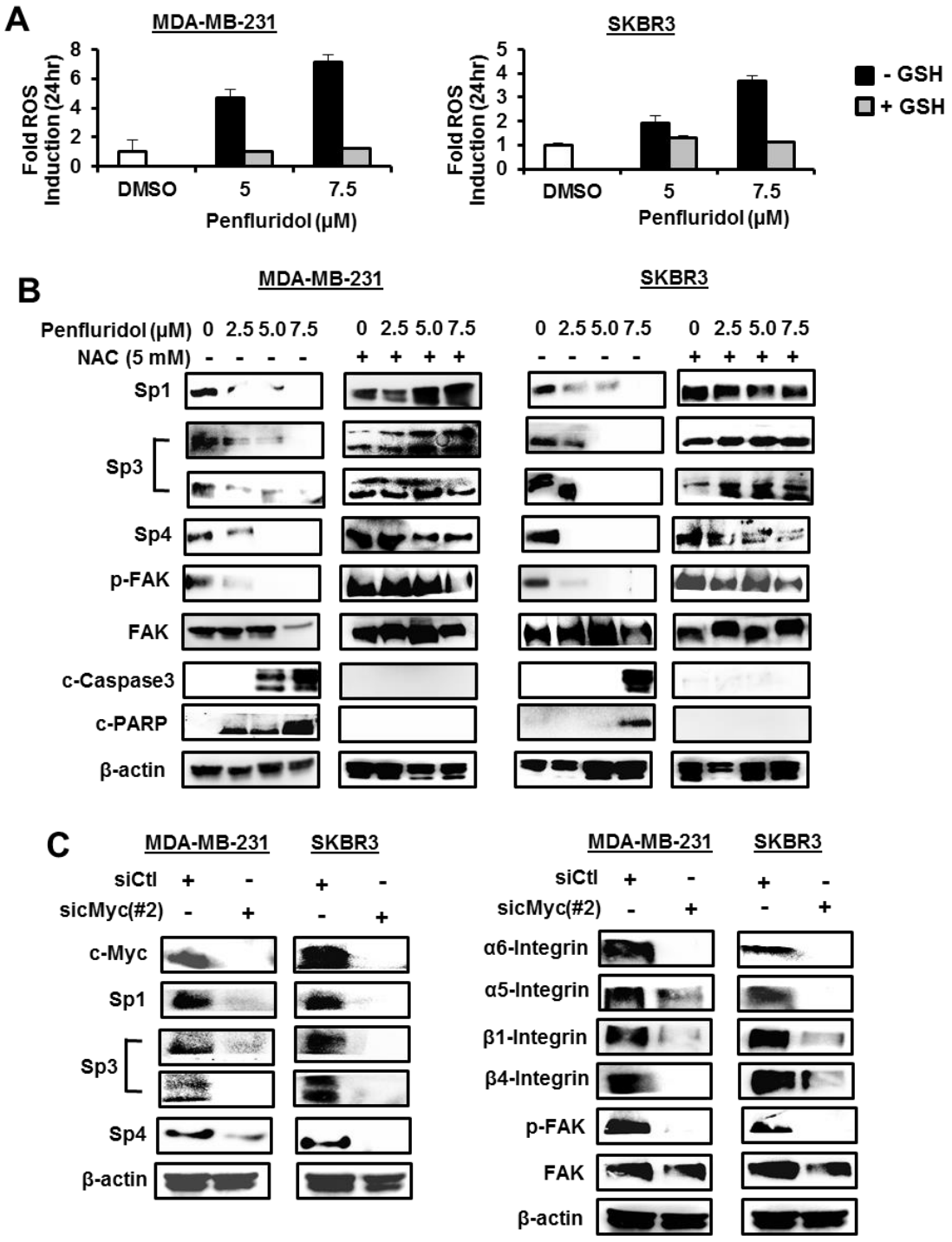
Supplemental Figure A-10. DIM-C-pPhOH inhibits β1-integrin and α5-integrin in pancreatic xenografts. Tumor lysates from an L3.6pL orthotopic xenograft experiment in vivo show that DIM-C-pPhOH (30 mg/kg/d) decreased expression of β1-integrin and α5-integrin compared to corn oil control. (Tumor lysates from 2 animals are shown.)

Supplemental Figure A-11



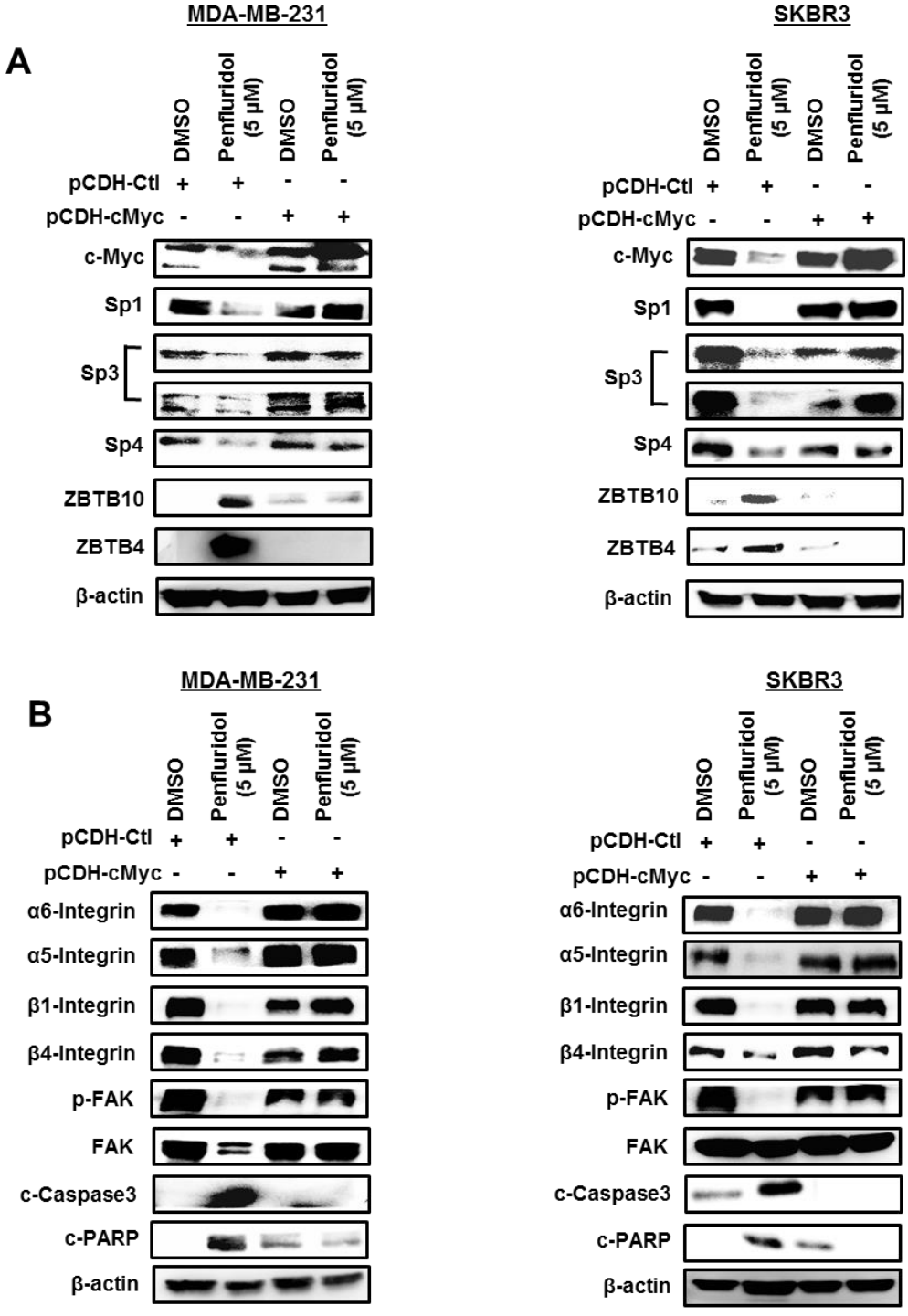
Supplemental Figure A-11. C-DIM inhibition of pancreatic and colon cancer cell migration is dependent on NR4A1 and β1-integrin. Pancreatic (A) and colon (B) cancer cells were treated with DMSO or 20 μM DIM-C-pPhOH alone or after transfection with siCtl (control), siNR4A1 or siβ1-integrin, and migration was determined in a Boyden chamber assay as outlined in the Materials and Methods. Results are expressed as means ± SE for 3 determinations per treatment group and significant ($p<0.05$) inhibition of migration is indicated (*).

Supplemental Figure A-12



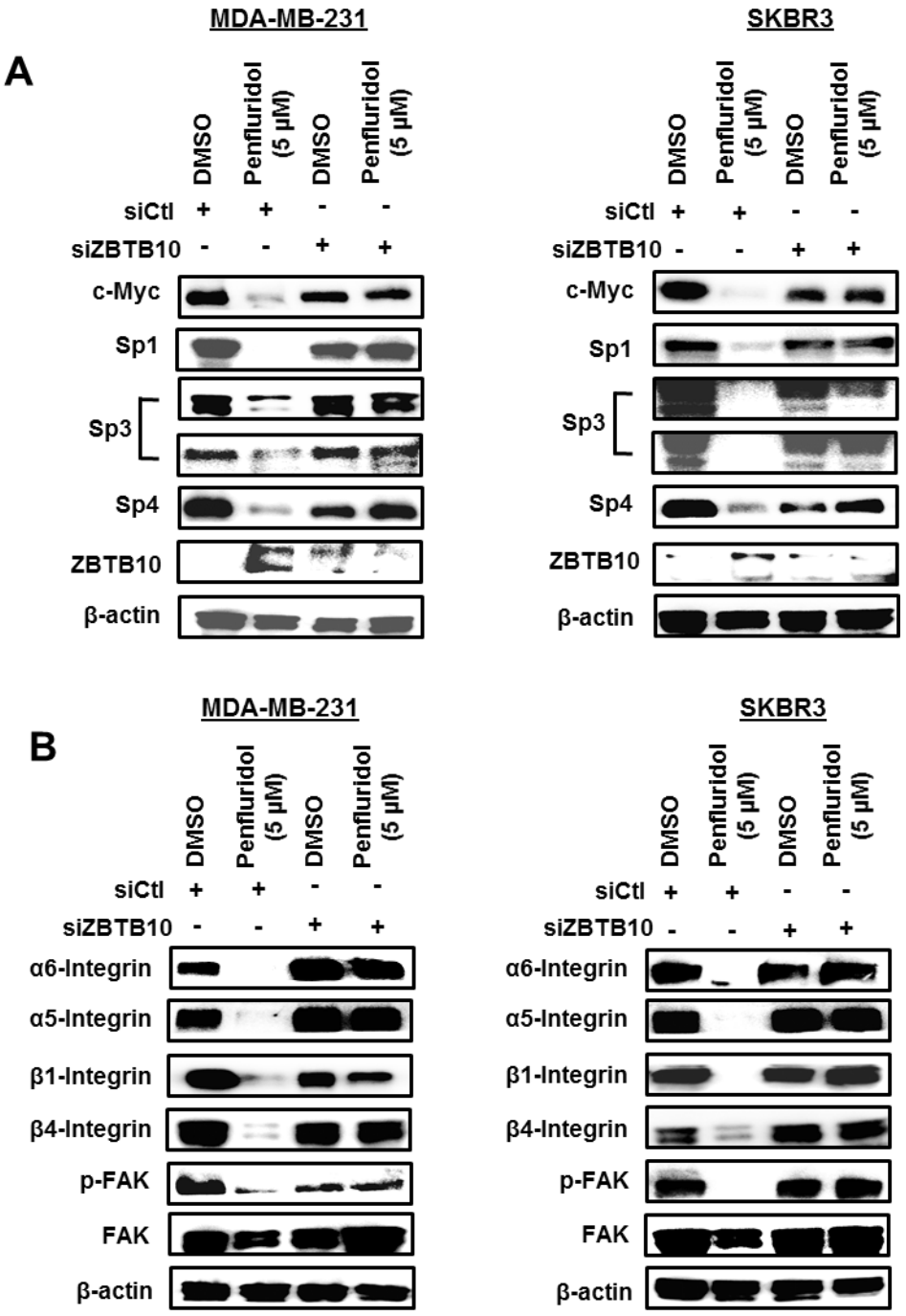
Supplemental Figure A-12. Role of ROS and cMyc in penfluridol-induced responses. (A) Induction of ROS. Cells were treated with penfluridol alone or in combination with GSH, and ROS was determined after 24 hr. (B) Role of ROS in penfluridol-induces responses. Cells were treated with penfluridol alone or in combination with NAC for 24 hr, and whole cell lysates were analyzed by western blots. (C) cMyc knockdown. Cells were transfected with siCtl (control) or siMyc(#2), and whole cell lysates were analyzed by western blots.

Supplemental Figure A-13



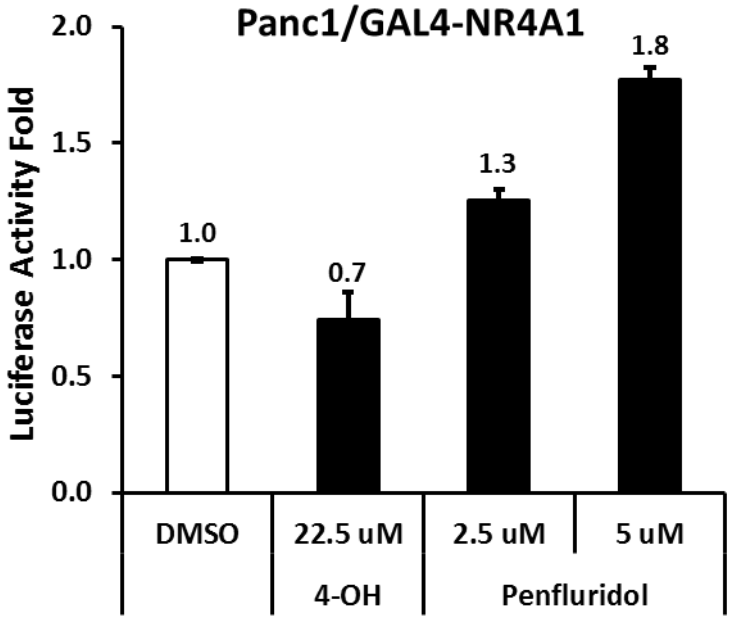
Supplemental Figure A-13. Myc expression rescues cells from penfluridol. Cells were treated with penfluridol or cMyc expression plasmid (pCDH-cMyc) or empty vector alone or in combination (A, B), and whole cell lysates were analyzed by western blots.

Supplemental Figure A-14



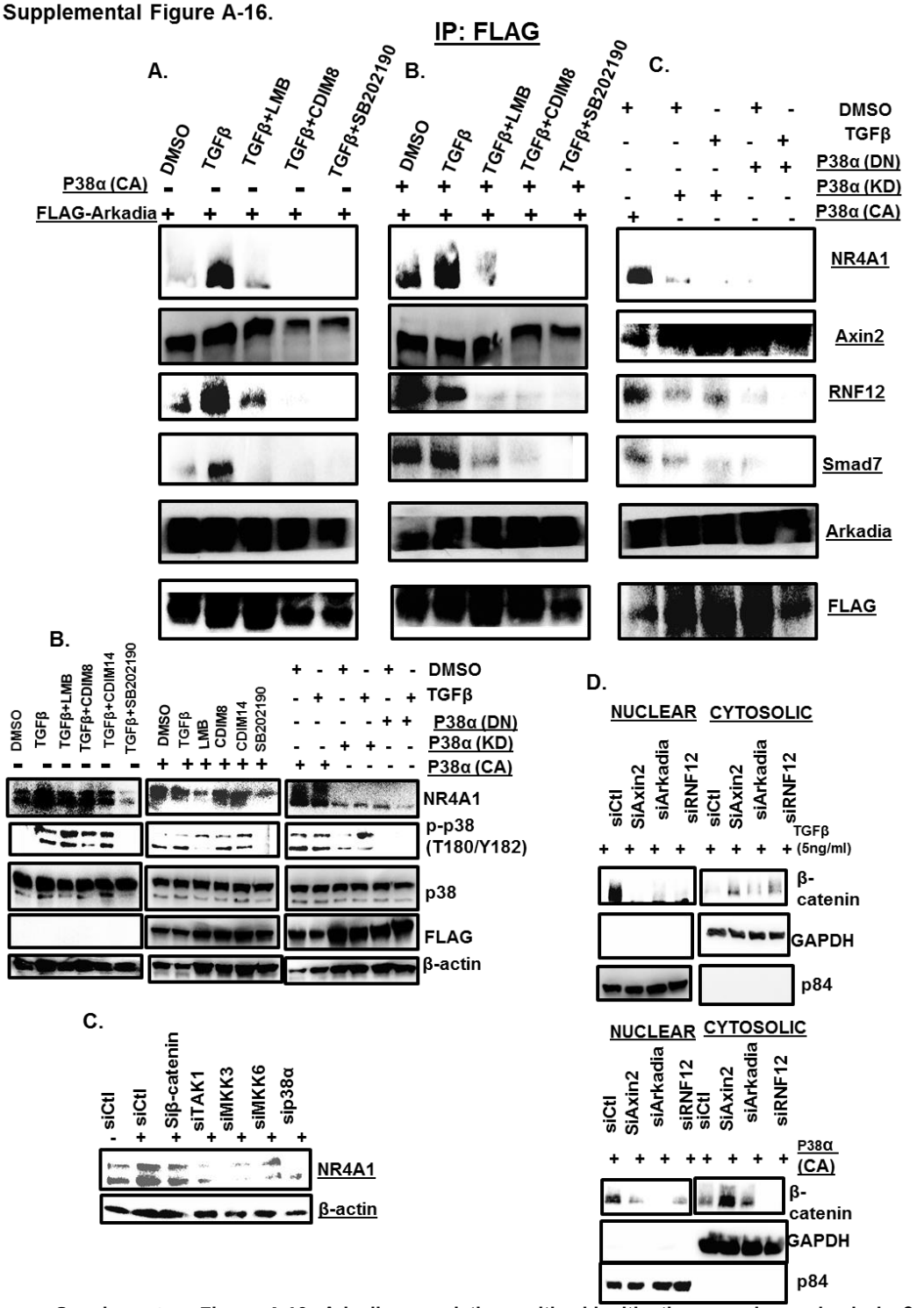
Supplemental Figure A-14. ZBTB10 knockdown rescues cells from penfluridol. Cells were treated with penfluridol or transfected with siZBTB10 or control oligonucleotide alone or in combination (A, B), and whole cell lysates were analyzed by western blots.

Supplementary Figure A-15



Supplementary Figure A-15. Penfluridol as an NR4A1 antagonist. Cells were treated with the NR4A1 antagonist DIM-C-pPhOH (4-OH) or penfluridol and transfected with a GAL4-NR4A1 chimera and reporter-luciferase construct (containing GAL4 response elements). Luciferase activity was determined (27). Only DIM-C-pPhOH decreased luciferase activity, characteristic of an NR4A antagonist (26, 27).

Supplemental Figure A-16.



Supplementary Figure A-16. Arkadia associations with ubiquitination complex and role in β-catenin localization. Pulldown of Arkadia demonstrates association with NR4A1 in the presence of TGFβ and p38α(CA) (A). TGFβ and p38α(CA) induce NR4A1 (B) and this induction is blocked by inhibiting kinases upstream of p38α (C). Knockdown of ubiquitination complex members promotes cytosolic sequestration of β-catenin (D).

APPENDIX B

Supplemental Table B1. Sp1-regulated associated with growth inhibition, cell death and inhibition of migration/invasion after Sp1 knockdown: expected and inversely regulated genes. This table as well as Supplemental B2 and B3 refer to Chapter VI.

A.

Expected				**Inversely related**			
decreased cell proliferation				**increased cell proliferation**			
up-genes (118)	fold change	**down genes (241)**	fold change	**up-genes (150)**	fold change	**down genes (120)**	fold change
IFITM1	12.322	**RRM2**	-7.791	**LMNA**	9.997	**CASP3**	-3.784
HMOX1	7.399	**VIM**	-5.703	**ISG15**	5.775	**NFIB**	-3.751
CEBPD	5.371	**DKK1**	-4.643	**C1QBP**	4.647	**SIRT2**	-3.049
SOD2	5.022	**EGR1**	-4.506	**CXCL2**	4.516	**SPRY1**	-2.903
OAS3	4.22	**FOS**	-4.319	**CBX7**	4.368	**TCF12**	-2.839
IFNL1	4.039	**PKM**	-4.043	**CX3CL1**	4.048	**ARRDC3**	-2.72
RNH1	4.007	**CCNB1**	-3.893	**ISG20**	3.98	**NFIX**	-2.667
MSX1	3.958	**CD44**	-3.853	**CCL5**	3.871	**RND3**	-2.651
GEMIN2	3.761	**BMI1**	-3.772	**BATF2**	3.571	**ABCC5**	-2.643
IFIT3	3.75	**DUSP6**	-3.68	**ETS1**	3.545	**TJP1**	-2.634
IL32	3.678	**CDK19**	-3.586	**RCE1**	3.201	**KLK3**	-2.632
ABCC3	3.603	**CITED2**	-3.579	**SET**	3.167	**EIF4G2**	-2.619
TIMP2	3.59	**PDE5A**	-3.301	**TNF**	3.013	**TRIB1**	-2.587
NPTX1	3.572	**CENPF**	-3.165	**NOTCH4**	2.904	**PSRC1**	-2.542
CXCL10	3.57	**KIF20A**	-3.119	**NOLC1**	2.76	**AKAP12**	-2.536
SERPINA3	3.451	**PXN**	-3.096	**SRPK2**	2.753	**NRP1**	-2.48
CD83	3.429	**CCND3**	-2.962	**UBD**	2.732	**SLC9A3R**	-2.433
TNFAIP3	3.066	**KIF11**	-2.927	**NDEL1**	2.707	**LIN9**	-2.367
CDCA4	2.909	**ASPM**	-2.887	**SERPINH1**	2.695	**KIF23**	-2.156
PTX3	2.81	**TOP2A**	-2.876	**EBI3**	2.681	**FRMD6**	-2.094
GPR56	2.782	**DDAH1**	-2.847	**CCL2**	2.649	**SPRY2**	-2.073
DACT3	2.685	**EOMES**	-2.712	**ANTXR1**	2.64	**KIF2C**	-2.066
GBP1	2.664	**SS18**	-2.698	**RAC2**	2.57	**PLOD2**	-2.063
LEPROT	2.637	**MAP3K7**	-2.693	**LRPAP1**	2.531	**CDKN3**	-2.053
COMMD5	2.596	**EGFR**	-2.562	**STAT5A**	2.468	**ASPH**	-2.036
CBL	2.56	**RAP1B**	-2.559	**TPD52**	2.428	**SRGAP2**	-1.99
STC1	2.49	**SLC2A1**	-2.53	**UPP1**	2.424	**SESN1**	-1.986
EIF1	2.471	**BIRC5**	-2.525	**S100A4**	2.414	**TGFBR2**	-1.966
TAP1	2.422	**CCNA2**	-2.515	**DIXDC1**	2.407	**WASPIP**	-1.943
IFNL2	2.42	**PRKCA**	-2.515	**PTPMT1**	2.395	**CBFB**	-1.929
PARP10	2.395	**HIF1A**	-2.482	**CYP1B1**	2.32	**HS.13003**	-1.927
PHLPP2	2.366	**BUB1**	-2.457	**CBS**	2.316	**RBL2**	-1.897
MUL1	2.343	**HMMR**	-2.446	**EMP1**	2.271	**PHC1**	-1.894
IRF1	2.31	**USP47**	-2.431	**SDC4**	2.252	**MIB1**	-1.861
HRASLS	2.294	**DLGAP5**	-2.398	**PTP4A3**	2.247	**CHES1**	-1.85
BECN1	2.285	**IRS1**	-2.37	**SHC1**	2.232	**HIPK2**	-1.85
RALBP1	2.277	**AURKA**	-2.368	**MVP**	2.184	**EGR2**	-1.842
PLSCR1	2.267	**PAPSS2**	-2.334	**BCL2L1**	2.147	**APBB2**	-1.837
RARRES3	2.248	**KIF20B**	-2.318	**NAP1L1**	2.135	**TFAM**	-1.823
TNFRSF9	2.211	**DICER1**	-2.301	**CTSD**	2.132	**GJA1**	-1.821
UCP2	2.179	**NEK2**	-2.297	**SUMO2**	2.117	**CCNG2**	-1.787

GAL	2.172	**CYR61**	-2.284	**MGAT3**	2.108	**RB1**	-1.786
TNFRSF14	2.162	**MAPK6**	-2.25	**YWHAG**	2.096	**TP53BP2**	-1.776
SIGIRR	2.162	**AGO2**	-2.238	**CCRK**	2.095	**TFPI**	-1.762
SOCS1	2.153	**CDCA8**	-2.234	**CD320**	2.078	**ITGA6**	-1.757
EI24	2.151	**PPIA**	-2.23	**EWSR1**	2.031	**ERRFI1**	-1.747
DAXX	2.144	**TESK1**	-2.215	**FSTL3**	2.028	**WNK2**	-1.738
PMP22	2.12	**STAT5B**	-2.211	**SRF**	2.018	**CAT**	-1.729
RASSF5	2.108	**P4HA1**	-2.201	**TBP**	2.002	**TRIM24**	-1.709
CAMK2N1	2.052	**SPTBN1**	-2.188	**ARHGEF2**	1.991	**ATG5**	-1.701
MAFG	2.038	**CDK1**	-2.184	**CORO1B**	1.99	**PKP2**	-1.697
GNE	2.014	**TPX2**	-2.147	**FEZ1**	1.98	**FOXF2**	-1.693
DNAJA3	2.011	**ERCC1**	-2.115	**NCOR2**	1.973	**RASA1**	-1.692
SMPD1	2.009	**PBK**	-2.106	**RALGDS**	1.953	**FBXO11**	-1.686
IFI16	2.008	**RPRD1B**	-2.098	**CCL20**	1.95	**SPRED1**	-1.685
SMARCA2	1.994	**TTK**	-2.084	**GBP2**	1.947	**HNRNPA1**	-1.675
TNFRSF1B	1.973	**YBX1**	-2.083	**IGF2BP3**	1.946	**USP10**	-1.672
LRRC32	1.969	**RGS2**	-2.075	**MAP1B**	1.943	**NDFIP1**	-1.67
IFITM3	1.962	**PLXNB2**	-2.066	**BCL3**	1.938	**KLHL13**	-1.667
TRAF1	1.959	**LRP5**	-2.036	**PSMA4**	1.934	**B3GNT2**	-1.666
MED25	1.934	**TACC3**	-2.036	**MYD88**	1.924	**HS.37160**	-1.659
IFNL3	1.923	**RAB22A**	-2.018	**SLC7A5**	1.913	**RAD17**	-1.653
NDRG4	1.898	**EGLN1**	-2.008	**CDK4**	1.911	**MAP2K1I**	-1.649
NUMA1	1.893	**ECT2**	-1.989	**STMN3**	1.908	**ENPP1**	-1.642
RPS6KA2	1.889	**PIK3R1**	-1.986	**ARF1**	1.903	**G3BP1**	-1.64
FBXO2	1.847	**EPAS1**	-1.985	**ATP5G1**	1.903	**TOP1**	-1.637
DIABLO	1.843	**FAT4**	-1.983	**ST5**	1.898	**STARD13**	-1.621
TRIM22	1.835	**PPP1R13L**	-1.979	**ID3**	1.898	**RAD21**	-1.618
NACC2	1.818	**KIF18A**	-1.976	**STX3**	1.895	**CTBP2**	-1.618
IGFBP6	1.813	**NFIA**	-1.971	**PRNP**	1.857	**FOXA2**	-1.607
EFNB3	1.812	**CDC20**	-1.966	**DCLRE1A**	1.85	**CMA1**	-1.602
DKK3	1.805	**TRIB2**	-1.943	**JAG1**	1.837	**NAB1**	-1.601
PCBP4	1.79	**TTLL4**	-1.935	**HMGB1**	1.811	**TES**	-1.594
QPCT	1.77	**NUDT1**	-1.917	**NACA**	1.806	**RANBP9**	-1.591
GADD45A	1.755	**CTGF**	-1.906	**NOD2**	1.794	**PLXNA3**	-1.583
NKX3-1	1.753	**NRARP**	-1.9	**ICAM1**	1.793	**RTN4**	-1.583
EPHA2	1.749	**TRPC1**	-1.889	**ABCG1**	1.77	**CASP7**	-1.582
PMAIP1	1.745	**LBR**	-1.886	**TAGLN2**	1.769	**MIR1-2**	-1.58
GDF15	1.736	**RPS6KB1**	-1.872	**LCN2**	1.764	**SMPD2**	-1.573
LIMK1	1.7	**CSNK1G3**	-1.862	**NCOA4**	1.763	**H2AFY**	-1.57
CCL3L3	1.698	**PTK2**	-1.858	**ALG13**	1.754	**PMEPA1**	-1.567
STAT2	1.692	**PIP4K2A**	-1.856	**LAP3**	1.742	**KLF10**	-1.566
NDUFS3	1.675	**CCDC88A**	-1.855	**LIF**	1.736	**CHEK2**	-1.562
SREBF1	1.672	**UIMC1**	-1.854	**ICOSLG**	1.731	**SEMA3A**	-1.561
ATF3	1.666	**LAMC1**	-1.854	**CSPG5**	1.73	**DAB2**	-1.559
B2M	1.665	**TAB2**	-1.852	**GCLC**	1.728	**SUV39H1**	-1.558
RAP1GAP	1.64	**MBP**	-1.848	**BAMBI**	1.726	**CTCF**	-1.557
ACTN4	1.638	**HGF**	-1.847	**PRMT5**	1.722	**SIAH1**	-1.554
PHF14	1.626	**ATMIN**	-1.845	**CDK6**	1.714	**COL4A1**	-1.553
LPIN1	1.619	**FRS2**	-1.838	**NFKB2**	1.707	**MERTK**	-1.553
BST2	1.612	**ERO1LB**	-1.824	**SKP2**	1.706	**SEC23A**	-1.553
IRX3	1.61	**TGIF1**	-1.822	**PSMB10**	1.705	**MIR203**	-1.552
INCA1	1.609	**FBXW11**	-1.808	**GPC1**	1.695	**ADORA3**	-1.55
TSC1	1.606	**RACGAP1**	-1.806	**ATM**	1.695	**MIR138-1**	-1.549

TP53I11	1.593	**ZIC2**	-1.803	**PPL**	1.688	**EMX2**	-1.541
ARHGDIB	1.58	**CDCA2**	-1.803	**IL15**	1.681	**APRIN**	-1.54
RHOB	1.579	**GBF1**	-1.793	**XBP1**	1.681	**TNFRSF8**	-1.538
JUND	1.579	**ANXA2**	-1.791	**MNAT1**	1.679	**CXADR**	-1.537
ZMPSTE24	1.569	**ITGA2**	-1.789	**NP**	1.677	**CNKSR1**	-1.535
SIRPA	1.566	**GNAI2**	-1.788	**PRKAR2A**	1.672	**PATZ1**	-1.533
NFKBIB	1.563	**FOXQ1**	-1.783	**CDC25B**	1.67	**P4HA2**	-1.533
LITAF	1.55	**PTGER4**	-1.781	**CXCL1**	1.667	**FAM188A**	-1.529
STAT1	1.549	**NFYA**	-1.777	**CISD1**	1.666	**CASK**	-1.526
BAG6	1.548	**NBN**	-1.766	**PLAT**	1.664	**TSPYL2**	-1.525
EEF1E1	1.546	**STAG1**	-1.763	**SLC25A4**	1.658	**MTBP**	-1.524
CEACAM1	1.545	**ZMYM2**	-1.762	**SNX12**	1.651	**SPRY4**	-1.519
UXT	1.544	**BLM**	-1.76	**MED28**	1.646	**CAST**	-1.518
NUP62	1.54	**HAS2**	-1.758	**SPTAN1**	1.644	**RUNX1**	-1.518
TSC22D3	1.539	**KPNA2**	-1.757	**MST1**	1.638	**BMP5**	-1.517
HSPA1A/HSPA1	1.527	**PTP4A2**	-1.746	**GPR177**	1.636	**ADRA1A**	-1.516
NUPR1	1.527	**PTPN3**	-1.744	**SRC**	1.634	**CD55**	-1.514
E2F7	1.524	**DVL3**	-1.744	**ESRRA**	1.621	**KRT7**	-1.512
SAT1	1.522	**RNF126**	-1.733	**DPYSL2**	1.618	**DUX4**	-1.512
BIN1	1.521	**SUV420H1**	-1.713	**ZFP36L1**	1.617	**IL1RN**	-1.51
PHB	1.515	**NCAPG**	-1.71	**WDR12**	1.613	**CAPRIN1**	-1.509
LRIG1	1.515	**PRPS2**	-1.705	**NFKB1**	1.611	**ARID4A**	-1.504
NAIF1	1.511	**H2AFX**	-1.703	**IL15RA**	1.606	**CNN2**	-1.502
AGTRAP	1.508	**PYCARD**	-1.702	**DYRK1B**	1.605	**BACH2**	-1.501
		MAD2L1	-1.7	**NOP58**	1.605	**CNTN1**	-1.5
		MKI67	-1.7	**PDCL3**	1.6	**PTPN2**	-1.5
		NRG1	-1.697	**TFE3**	1.593		
		INO80	-1.694	**CNPY2**	1.59		
		ELMO2	-1.693	**USP18**	1.59		
		TYMS	-1.692	**NFKBIA**	1.588		
		ABL1	-1.689	**TXLNA**	1.588		
		FOXM1	-1.688	**RAB33A**	1.583		
		POLR3E	-1.687	**DUSP12**	1.577		
		NFAT5	-1.686	**TSPAN3**	1.577		
		RAN	-1.683	**MYH14**	1.562		
		NCOA2	-1.683	**POLR2L**	1.56		
		NDRG1	-1.68	**PDCD10**	1.558		
		KCNMA1	-1.677	**PPP1R15A**	1.557		
		YBX3	-1.676	**ALS2**	1.556		
		FGFR4	-1.675	**TNFSF10**	1.555		
		NFS1	-1.674	**CD58**	1.546		
		HGS	-1.672	**TNFSF9**	1.545		
		AGO4	-1.67	**COPS2**	1.534		
		BCAR3	-1.666	**TNFAIP8**	1.529		
		PLAG1	-1.666	**SLC3A2**	1.529		
		NASP	-1.661	**TOP1MT**	1.525		
		GAD1	-1.659	**VEGFB**	1.519		
		MALT1	-1.656	**VGF**	1.517		
		DOCK1	-1.656	**TRNP1**	1.517		
		PAK2	-1.654	**RNASEH2B**	1.516		
		DVL2	-1.653	**BCL2A1**	1.515		
		AKR1C1/AKR1C	-1.652	**CLN3**	1.514		

		ATAD2	-1.651	**CDC42**	1.512		
		PRKAA1	-1.651	**AKIRIN2**	1.508		
		KMT2C	-1.651	**RCAN1**	1.506		
		ACVR2A	-1.648	**CDK5R1**	1.502		
		JAK2	-1.642				
		PDK1	-1.638				
		ROR1	-1.638				
		TXNDC5	-1.637				
		YAP1	-1.632				
		F2RL1	-1.631				
		NR2C2	-1.63				
		LSM1	-1.63				
		GPN3	-1.629				
		USP7	-1.627				
		CDC45	-1.619				
		SLC29A1	-1.618				
		PAK4	-1.617				
		ARFGAP3	-1.603				
		BUB3	-1.602				
		MAPK10	-1.602				
		DIDO1	-1.601				
		LDHA	-1.6				
		PTPN13	-1.6				
		F2R	-1.599				
		PTTG1	-1.595				
		ACTB	-1.593				
		AURKB	-1.593				
		KIF2A	-1.591				
		MSRA	-1.59				
		CAMK4	-1.589				
		WWTR1	-1.588				
		MCM4	-1.587				
		LDLRAP1	-1.586				
		PLK1	-1.583				
		BAIAP2L1	-1.583				
		DHFR	-1.581				
		AKT2	-1.579				
		NEDD4L	-1.578				
		TEAD4	-1.577				
		B4GALT6	-1.575				
		KRAS	-1.573				
		AR	-1.57				
		TBX1	-1.567				
		PDGFC	-1.566				
		SERPINB9	-1.566				
		PCGF2	-1.561				
		IL13RA1	-1.561				
		ITGB1	-1.558				
		LIN28B	-1.551				
		NTS	-1.549				
		CHN1	-1.545				
		POLH	-1.543				

		KDM5B	-1.541				
		E2F8	-1.54				
		IL2RG	-1.539				
		AGO3	-1.538				
		TP63	-1.537				
		CREB1	-1.536				
		ID4	-1.534				
		LCK	-1.534				
		CIZ1	-1.534				
		SMN1/SMN2	-1.533				
		CTTN	-1.532				
		SH3KBP1	-1.531				
		EPS8	-1.53				
		MCM10	-1.53				
		MCL1	-1.529				
		AKT3	-1.527				
		AKIRIN1	-1.525				
		CAMKK1	-1.524				
		AFAP1	-1.522				
		TCF4	-1.521				
		DDX17	-1.52				
		RTKN2	-1.519				
		HOXB3	-1.518				
		SGK1	-1.516				
		MEF2C	-1.515				
		CDC16	-1.514				
		FGF10	-1.514				
		ABCC9	-1.513				
		CDT1	-1.513				
		CALB1	-1.51				
		RIPK1	-1.509				
		PKP3	-1.509				
		PIK3R3	-1.508				
		ACSL4	-1.508				
		UBE2C	-1.508				
		NUP98	-1.508				
		mir-154	-1.507				
		VASP	-1.507				
		RAF1	-1.504				
		VEGFC	-1.503				
		ST6GAL1	-1.502				
		F11R	-1.501				
		CHEK1	-1.5				

B.

Expected				**Inversely related**			
Increased cell death				**Decreased cell death**			
Up-genes (140)	Fold change	**Down genes**	Fold change	**Up-genes (149)**	Fold change	**Down genes**	Fold change
ISG15	5.775	**RRM2**	-7.791	**HMOX1**	7.399	**NME4**	-5.372

OAS1	5.763	**STK40**	-4.272	**IFI6**	5.463	**DKK1**	-4.643
CEBPD	5.371	**PKM2**	-4.043	**SOD2**	5.022	**EGR1**	-4.506
MX1	4.743	**SLC4A7**	-3.856	**CXCL2**	4.516	**FOS**	-4.319
OAS3	4.220	**CD44**	-3.853	**LGALS3B**	4.241	**CCNB1**	-3.893
CX3CL1	4.048	**BMI1**	-3.772	**IL29**	4.039	**CASP3**	-3.784
NPTX1	3.572	**NFIB**	-3.751	**MSX1**	3.958	**DUSP6**	-3.680
CXCL10	3.570	**CITED2**	-3.579	**STX8**	3.903	**PDE5A**	-3.301
ETS1	3.545	**DEK**	-3.429	**CCL5**	3.871	**SIRT2**	-3.049
HS1BP3	3.536	**PCNA**	-3.224	**SIP1**	3.761	**DNASE1L1**	-3.038
IFIH1	3.332	**CENPF**	-3.165	**IFIT3**	3.750	**TOP2A**	-2.876
SMARCC1	3.297	**PXN**	-3.096	**IL32**	3.678	**TCF12**	-2.839
SET	3.167	**CCND3**	-2.962	**ABCC3**	3.603	**TFDP2**	-2.646
TNF	3.013	**KIF11**	-2.927	**TIMP2**	3.590	**APOBEC3B**	-2.518
NOTCH4	2.904	**EOMES**	-2.712	**SERPINA3**	3.451	**HMMR**	-2.446
NFKBIZ	2.775	**MAP3K7**	-2.693	**SELE**	3.414	**SPAG5**	-2.398
SRPK2	2.753	**NFIX**	-2.667	**TUB**	3.155	**ANTXR2**	-2.383
UBD	2.732	**RND3**	-2.651	**TNFAIP3**	3.066	**SLC25A24**	-2.320
BOK	2.709	**ABCC5**	-2.643	**HSPBP1**	2.927	**ZFYVE16**	-2.307
CASP4	2.672	**KLK3**	-2.632	**DAG1**	2.771	**ZAK**	-2.283
ANTXR1	2.640	**EIF4G2**	-2.619	**NOLC1**	2.760	**CCDC109A**	-2.260
ADORA2A	2.600	**TRIB1**	-2.587	**NDEL1**	2.707	**ATP2A2**	-2.192
RAC2	2.570	**EGFR**	-2.562	**HAND1**	2.707	**SPTBN1**	-2.188
STC1	2.490	**RAP1B**	-2.559	**SERPINH1**	2.695	**CDC2**	-2.184
ZC3H12A	2.471	**PON2**	-2.548	**CCL2**	2.649	**UACA**	-2.156
ITGB3BP	2.384	**AKAP12**	-2.536	**CBL**	2.560	**FRMD6**	-2.094
CYP1B1	2.320	**SLC2A1**	-2.530	**LRPAP1**	2.531	**GULP1**	-2.088
IRF1	2.310	**BIRC5**	-2.525	**PCTP**	2.523	**TTK**	-2.084
HRASLS	2.294	**PRKCA**	-2.515	**STAT5A**	2.468	**CKAP2**	-2.054
BECN1	2.285	**HIF1A**	-2.482	**TRIAP1**	2.438	**CDKN3**	-2.053
SP110	2.277	**NRP1**	-2.480	**TPD52**	2.428	**LRP5**	-2.036
EMP1	2.271	**USP47**	-2.431	**IL28A**	2.420	**ELF4**	-2.022
PLSCR1	2.267	**NEK6**	-2.426	**S100A4**	2.414	**RAB22A**	-2.018
PI3	2.266	**NDC80**	-2.415	**PSMB8**	2.407	**FAF1**	-1.993
MYH9	2.255	**KIF14**	-2.384	**PTPMT1**	2.395	**ECT2**	-1.989
SDC4	2.252	**IRS1**	-2.370	**ACO2**	2.371	**SNAP25**	-1.980
SHC1	2.232	**AURKA**	-2.368	**LMO4**	2.318	**CDC20**	-1.966
TNFRSF9	2.211	**SOX21**	-2.322	**CBS**	2.316	**WASPIP**	-1.943
MVP	2.184	**DICER1**	-2.301	**GBA**	2.230	**TRIB2**	-1.943
DAXX	2.144	**NEK2**	-2.297	**GAL**	2.172	**TRAF7**	-1.941
IFIT2	2.121	**CYR61**	-2.284	**SIGIRR**	2.162	**HS.130036**	-1.927
PMP22	2.120	**EIF2C2**	-2.238	**SOCS1**	2.153	**ST3GAL3**	-1.914
RNF13	2.118	**PPIA**	-2.230	**EI24**	2.151	**TRPS1**	-1.913
FSTL3	2.028	**STAT5B**	-2.211	**BCL2L1**	2.147	**FBXO32**	-1.910
DNAJA3	2.011	**CENPE**	-2.197	**WFS1**	2.137	**SCP2**	-1.900
SMPD1	2.009	**CCT3**	-2.181	**MGAT3**	2.108	**FAM72A**	-1.875
IFI16	2.008	**TUBB3**	-2.150	**YWHAG**	2.096	**BUB1B**	-1.867
PRELID1	2.005	**TPX2**	-2.147	**EBAG9**	2.091	**MIB1**	-1.861
SMARCA2	1.994	**DHRS2**	-2.132	**ECGF1**	2.090	**CCDC88A**	-1.855
LAMP1	1.975	**NT5E**	-2.120	**OSCAR**	2.088	**HIPK2**	-1.850
TNFRSF1	1.973	**SRI**	-2.120	**BEX2**	2.082	**MBP**	-1.848
NCOR2	1.973	**ERCC1**	-2.115	**PINK1**	2.062	**XPR1**	-1.842
MAP3K8	1.958	**PBK**	-2.106	**WDR4**	2.045	**RFK**	-1.832

MAP1B	1.943	**SORBS2**	-2.105	**MAFG**	2.038	**TIA1**	-1.822
XAF1	1.940	**CCT5**	-2.088	**EWSR1**	2.031	**CDC25C**	-1.810
MYD88	1.924	**YBX1**	-2.083	**MAD2L2**	2.028	**CABLES2**	-1.799
CDK4	1.911	**SPRY2**	-2.073	**RGS10**	2.022	**MAP3K9**	-1.785
ID3	1.898	**STIL**	-2.041	**SRF**	2.018	**PTGER4**	-1.781
PARP14	1.897	**TACC3**	-2.036	**TBP**	2.002	**TP53BP2**	-1.776
NUMA1	1.893	**EGLN1**	-2.008	**ARHGEF2**	1.991	**STAG1**	-1.763
RPS6KA2	1.889	**HIPK3**	-1.993	**TAOK3**	1.978	**TFPI**	-1.762
OGDH	1.888	**PIK3R1**	-1.986	**TRAF1**	1.959	**BLM**	-1.760
IRAK2	1.882	**EPAS1**	-1.985	**BCL3**	1.938	**ITGA6**	-1.757
PRNP	1.857	**FAT4**	-1.983	**CAMK2G**	1.920	**TSC22D2**	-1.750
DIABLO	1.843	**MED29**	-1.983	**PLEC1**	1.916	**MELK**	-1.729
PTRH2	1.836	**PPP1R13L**	-1.979	**NUAK1**	1.890	**MCM7**	-1.723
CANX	1.829	**TGFBR2**	-1.966	**SCYL1**	1.861	**CD69**	-1.707
SFT2D2	1.821	**CENPA**	-1.965	**STXBP1**	1.850	**H2AFX**	-1.703
NACC2	1.818	**CKAP5**	-1.947	**FBXO2**	1.847	**PYCARD**	-1.702
IGFBP6	1.813	**CBFB**	-1.929	**MAPKAP1**	1.844	**ATG5**	-1.701
HMGB1	1.811	**NUDT1**	-1.917	**GCLM**	1.837	**MAD2L1**	-1.700
DKK3	1.805	**GPHN**	-1.909	**JAG1**	1.837	**HS.551128**	-1.697
ICAM1	1.793	**CTGF**	-1.906	**BCL2L2**	1.837	**FOXF2**	-1.693
OLR1	1.786	**RBL2**	-1.897	**BFAR**	1.800	**RASA1**	-1.692
IRF7	1.774	**TRPC1**	-1.889	**MEF2D**	1.797	**ABL1**	-1.689
TGFBI	1.772	**FBXO5**	-1.873	**SH3GLB1**	1.795	**FGFR4**	-1.675
LCN2	1.764	**RPS6KB1**	-1.872	**PEA15**	1.779	**PPP2R5A**	-1.660
B4GALT5	1.760	**PTK2**	-1.858	**SLC40A1**	1.779	**HS.371609**	-1.659
GADD45A	1.755	**UIMC1**	-1.854	**ABCG1**	1.770	**MALT1**	-1.656
NKX3-1	1.753	**MAP3K7IP2**	-1.852	**TAGLN2**	1.769	**DOCK1**	-1.656
CMIP	1.751	**HGF**	-1.847	**NCOA4**	1.763	**PAK2**	-1.654
EPHA2	1.749	**ATMIN**	-1.845	**HLA-F**	1.761	**SDC1**	-1.638
EIF2S1	1.747	**PIAS2**	-1.844	**NAGLU**	1.760	**PDK1**	-1.638
PMAIP1	1.745	**EGR2**	-1.842	**GDF15**	1.736	**TOP1**	-1.637
LIF	1.736	**HS.334831**	-1.838	**ICOSLG**	1.731	**ATN1**	-1.632
CSTB	1.725	**TFAM**	-1.823	**GCLC**	1.728	**PERP**	-1.632
CDK6	1.714	**CDCP1**	-1.816	**PRMT5**	1.722	**F2RL1**	-1.631
SHISA5	1.709	**RACGAP1**	-1.806	**CLCN7**	1.709	**USP7**	-1.627
DRAM1	1.708	**CDCA2**	-1.803	**NFKB2**	1.707	**RAD21**	-1.618
CCL3L3	1.698	**GJB2**	-1.796	**SKP2**	1.706	**SLC29A1**	-1.618
GPC1	1.695	**ANXA2**	-1.791	**PSMB10**	1.705	**REPS2**	-1.616
ATM	1.695	**ITGA2**	-1.789	**CYB5A**	1.699	**TLR1**	-1.609
STAT2	1.692	**RB1**	-1.786	**HBXIP**	1.690	**STAP2**	-1.607
IL15	1.681	**NFYA**	-1.777	**XBP1**	1.681	**MAPK10**	-1.602
SREBF1	1.672	**NSF**	-1.777	**MNAT1**	1.679	**LDHA**	-1.600
ZNF622	1.668	**TBL1XR1**	-1.771	**NP**	1.677	**PTPN13**	-1.600
ATF3	1.666	**NBN**	-1.766	**RPS24**	1.672	**F2R**	-1.599
B2M	1.665	**ZMYM2**	-1.762	**ARID3B**	1.668	**PTTG1**	-1.595
PLAT	1.664	**HAS2**	-1.758	**CXCL1**	1.667	**RANBP9**	-1.591
RFXANK	1.658	**KPNA2**	-1.757	**BCL2L13**	1.664	**CAMK4**	-1.589
SLC25A4	1.658	**PTP4A2**	-1.746	**CHMP4B**	1.660	**RTN4**	-1.583
BRMS1	1.657	**HADHA**	-1.746	**RAB32**	1.658	**CASP7**	-1.582
HRASLS3	1.645	**SPG7**	-1.740	**MED28**	1.646	**CCBL1**	-1.581
ADI1	1.638	**PSIP1**	-1.734	**RAP1GAP**	1.640	**MIR1-2**	-1.580
PPP1R15	1.631	**FBXL5**	-1.733	**MST1**	1.638	**SLAMF6**	-1.579

EFHC1	1.627	**DMD**	-1.731	**DDAH2**	1.635	**KRAS**	-1.573
MLKL	1.625	**CAT**	-1.729	**SRC**	1.634	**SMPD2**	-1.573
MLLT11	1.620	**TOP2B**	-1.719	**STRADB**	1.623	**AR**	-1.570
PRR7	1.617	**SERP1**	-1.715	**INTS1**	1.619	**PMEPA1**	-1.567
AKAP1	1.614	**TRIM24**	-1.709	**NFKB1**	1.611	**CHEK2**	-1.562
SDHC	1.613	**GLUD1**	-1.705	**IL15RA**	1.606	**SEMA3A**	-1.561
DUSP2	1.612	**MKI67**	-1.700	**TSC1**	1.606	**DAB2**	-1.559
TP53I11	1.593	**TYMS**	-1.692	**FLOT2**	1.605	**ITGB1**	-1.558
CNPY2	1.590	**NUPL1**	-1.691	**APH1B**	1.604	**SIAH1**	-1.554
CTSL1	1.587	**FOXM1**	-1.688	**DNAJA1**	1.599	**MIR203**	-1.552
MTCP1	1.583	**NFAT5**	-1.686	**USP18**	1.590	**ADORA3**	-1.550
ARHGDIB	1.580	**NCOA2**	-1.683	**DDIT4**	1.589	**MIR138-1**	-1.549
RHOB	1.579	**NDRG1**	-1.680	**NFKBIA**	1.588	**CAPN10**	-1.545
DEDD2	1.569	**KCNMA1**	-1.677	**JUND**	1.579	**POLH**	-1.543
SIRPA	1.566	**CSDA**	-1.676	**SNCB**	1.568	**IL2RG**	-1.539
NFKBIB	1.563	**CCT6A**	-1.676	**UBQLN1**	1.560	**TNFRSF8**	-1.538
RBM43	1.558	**PLK4**	-1.674	**PDCD10**	1.558	**DEPDC1**	-1.537
PPP1R15	1.557	**USP10**	-1.672	**ALS2**	1.556	**ZMYND11**	-1.536
TNFSF10	1.555	**EIF2C4**	-1.670	**WDR68**	1.555	**CNKSR1**	-1.535
STAT1	1.549	**TNFRSF10**	-1.659	**IFNGR2**	1.554	**RRM1**	-1.534
BAT3	1.548	**DPP8**	-1.656	**ATP5S**	1.553	**SH2D4A**	-1.534
EEF1E1	1.546	**SPC25**	-1.655	**CEACAM1**	1.545	**PRKDC**	-1.533
TNFSF9	1.545	**FIGNL1**	-1.652	**UXT**	1.544	**SH3KBP1**	-1.531
HCST	1.542	**ATAD2**	-1.651	**NFE2L1**	1.544	**SCRIB**	-1.530
CHMP5	1.542	**PRKAA1**	-1.651	**VOPP1**	1.542	**AFAP1**	-1.522
MCOLN2	1.542	**WEE1**	-1.646	**MBOAT7**	1.541	**RUNX1**	-1.518
NUPR1	1.527	**TPM3**	-1.646	**NUP62**	1.540	**PCDHGA3**	-1.514
HTATIP2	1.525	**CCNI**	-1.643	**TSC22D3**	1.539	**CDT1**	-1.513
SAT1	1.522	**JAK2**	-1.642	**BCL2L12**	1.536	**RIPK1**	-1.509
BIN1	1.521	**CES1**	-1.640	**TNFAIP8**	1.529	**ULBP3**	-1.508
VGF	1.517	**HS.128753**	-1.638	**SLC3A2**	1.529	**ING3**	-1.506
CDC42	1.512	**TXNDC5**	-1.637	**HSPA1B**	1.527	**SELL**	-1.506
TMEM158	1.506	**BCKDK**	-1.637	**HSPE1**	1.527	**TRIM13**	-1.506
SNN	1.500	**ZNF184**	-1.634	**E2F7**	1.524	**PHF17**	-1.503
		YAP1	-1.632	**GRINA**	1.524	**BACH2**	-1.501
		HLTF	-1.632	**VEGFB**	1.519	**PTPN2**	-1.500
		CASC5	-1.631	**PI4KB**	1.517		
		NR2C2	-1.630	**BCL2A1**	1.515		
		CDC45L	-1.619	**PHB**	1.515		
		DPYD	-1.618	**CLN3**	1.514		
		CIT	-1.618	**TNIP2**	1.507		
		ELMO1	-1.618	**RCAN1**	1.506		
		RAD18	-1.618	**DAD1**	1.503		
		CTBP2	-1.618	**CDK5R1**	1.502		
		PAK4	-1.617				
		FOXA2	-1.607				
		DIDO1	-1.601				
		LGMN	-1.586				
		PLK1	-1.583				
		AKT2	-1.579				
		NCAPG2	-1.577				
		PDGFC	-1.566				

		SERPINB9	-1.566				
		CDC14A	-1.562				
		EDNRB	-1.558				
		CTCF	-1.557				
		CASZ1	-1.555				
		ASAH2	-1.554				
		COL4A1	-1.553				
		MERTK	-1.553				
		NTS	-1.549				
		FOXB1	-1.545				
		BLVRA	-1.543				
		SP3	-1.542				
		EMX2	-1.541				
		GABRR1	-1.540				
		E2F8	-1.540				
		EXOC2	-1.538				
		EIF2C3	-1.538				
		TP73L	-1.537				
		CXADR	-1.537				
		CREB1	-1.536				
		SMN1	-1.533				
		CTTN	-1.532				
		MCM10	-1.530				
		MIR135A2	-1.529				
		MCL1	-1.529				
		AKT3	-1.527				
		ABCC4	-1.524				
		MTBP	-1.524				
		CAMKK1	-1.524				
		CP	-1.524				
		PDE4B	-1.522				
		TCF4	-1.521				
		LRP2	-1.519				
		NHEJ1	-1.519				
		CAST	-1.518				
		BMP5	-1.517				
		ABCE1	-1.517				
		SGK1	-1.516				
		ADRA1A	-1.516				
		MEF2C	-1.515				
		CLEC5A	-1.515				
		FGF10	-1.514				
		SIAH2	-1.514				
		CD55	-1.514				
		RABGGTB	-1.511				
		VPS13A	-1.511				
		IL1RN	-1.510				
		CALB1	-1.510				
		NOVA1	-1.509				
		CAPRIN1	-1.509				
		PKP3	-1.509				
		ACSL4	-1.508				

		UBE2C	-1.508				
		DNAJB1	-1.507				
		MIR655	-1.507				
		VASP	-1.507				
		PARD3	-1.505				
		HS.24119	-1.505				
		RAF1	-1.504				
		PDZK1	-1.503				
		VEGFC	-1.503				
		ST6GAL1	-1.502				
		NEK1	-1.502				
		MOBKL2C	-1.501				
		CHEK1	-1.500				

C.

Expected				**Inversely related**			
decreased migration				**increased migration**			
up-genes (19)	**increased migration**	**down genes (65)**	fold change	**up-genes (36)**	fold change	**down genes (19)**	fold change
HMOX1	7.399	**VIM**	-5.703	**SOD2**	5.022	**ARRDC3**	-2.72
TIMP2	3.59	**EGR1**	-4.506	**C1QBP**	4.647	**NRP1**	-2.48
EBI3	2.681	**CD44**	-3.853	**CX3CL1**	4.048	**IRS1**	-2.37
MYH9	2.255	**BMI1**	-3.772	**CCL5**	3.871	**SPRY2**	-2.073
CMTM8	2.139	**PXN**	-3.096	**CXCL10**	3.57	**FAF1**	-1.993
IFIT2	2.121	**TCF12**	-2.839	**ETS1**	3.545	**GJA1**	-1.821
DNAJA3	2.011	**MAP3K7**	-2.693	**DPAGT1**	3.026	**CAT**	-1.729
IL15	1.681	**EGFR**	-2.562	**TNF**	3.013	**GNAI1**	-1.705
ATF3	1.666	**RAP1B**	-2.559	**CCL2**	2.649	**RASA1**	-1.692
BRMS1	1.657	**SLC2A1**	-2.53	**S100A4**	2.414	**NDRG1**	-1.68
RAP1GAP	1.64	**PRKCA**	-2.515	**PTP4A3**	2.247	**TPM3**	-1.646
DPYSL2	1.618	**HIF1A**	-2.482	**SHC1**	2.232	**MIR1-2**	-1.58
TSC1	1.606	**HMMR**	-2.446	**MAP3K8**	1.958	**CHST10**	-1.578
ARHGDIB	1.58	**SLC9A3R1**	-2.433	**CCL20**	1.95	**SEMA3A**	-1.561
SIRPA	1.566	**AURKA**	-2.368	**STMN3**	1.908	**TP73L**	-1.537
TNFSF10	1.555	**CYR61**	-2.284	**ARF1**	1.903	**FAM188A**	-1.529
STAT1	1.549	**ECT2**	-1.989	**IGFBP6**	1.813	**MTBP**	-1.524
HTATIP2	1.525	**CTGF**	-1.906	**EFNB3**	1.812	**LAMC2**	-1.509
GRINA	1.524	**PPFIA1**	-1.889	**EPHA2**	1.749	**VASP**	-1.507
		PTK2	-1.858	**GDF15**	1.736		
		CCDC88A	-1.855	**PRMT5**	1.722		
		HGF	-1.847	**LIMK1**	1.7		
		CDCP1	-1.816	**CDC25B**	1.67		
		ANXA2	-1.791	**HRASLS3**	1.645		
		ITGA2	-1.789	**ACTN4**	1.638		
		FOXQ1	-1.783	**MST1**	1.638		
		PTGER4	-1.781	**SRC**	1.634		
		HAS2	-1.758	**ESRRA**	1.621		
		KPNA2	-1.757	**IL15RA**	1.606		
		ITGA6	-1.757	**DYRK1B**	1.605		
		PTP4A2	-1.746	**RHOB**	1.579		

		SSH1	-1.708	**TNFAIP8**	1.529		
		NRG1	-1.697	**VEGFB**	1.519		
		FOXM1	-1.688	**VGF**	1.517		
		BCAR3	-1.666	**PHB**	1.515		
		DOCK1	-1.656	**CDC42**	1.512		
		PAK2	-1.654				
		DVL2	-1.653				
		JAK2	-1.642				
		PDK1	-1.638				
		ROR1	-1.638				
		F2RL1	-1.631				
		ELMO1	-1.618				
		CTBP2	-1.618				
		LMO7	-1.618				
		DIDO1	-1.601				
		F2R	-1.599				
		PTTG1	-1.595				
		RANBP9	-1.591				
		WWTR1	-1.588				
		KRAS	-1.573				
		AR	-1.57				
		DAB2	-1.559				
		RAP2A	-1.559				
		ITGB1	-1.558				
		MERTK	-1.553				
		LIN28B	-1.551				
		LCK	-1.534				
		CTTN	-1.532				
		AKT3	-1.527				
		AFAP1	-1.522				
		FGF10	-1.514				
		ACSL4	-1.508				
		VEGFC	-1.503				
		ST6GAL1	-1.502				

Supplemental Table B2. Sp3-regulated associated with growth inhibition, cell death and inhibition of migration/invasion after Sp3 knockdown: expected and inversely regulated genes.

A.

Expected				**Inversely related**			
decreased cell proliferation				**increased cell proliferation**			
up-genes (58)	fold change	**down genes (429)**	fold change	**up-genes (112)**	fold change	**down genes (244)**	fold change
TXNIP	5.257	**EGR1**	-15.086	**LMNA**	12.109	**TRIB1**	-4.97
SREBF1	4.275	**RAC1**	-8.138	**CBX7**	4.092	**CAST**	-4.411
CDCA4	3.527	**RRM2**	-7.011	**SET**	3.643	**RNF144**	-4.078
RNH1	3.391	**IL8**	-6.204	**C1QBP**	3.473	**STAT1**	-4.047
HMGB3	3.034	**BIRC3**	-6.182	**DEPDC6**	3.277	**TNFRSF21**	-4.036
UCP2	2.945	**FOS**	-5.573	**TFF3**	3.153	**STEAP3**	-3.66

SIP1	2.815	**LAMC1**	-5.183	**HMGB2**	2.987	**PHLDA1**	-3.657
PCBP4	2.509	**CTGF**	-4.591	**HMGB1**	2.955	**ARRDC3**	-3.535
FBXO2	2.378	**PLAU**	-4.394	**LRPAP1**	2.864	**EGR2**	-3.392
SIGIRR	2.242	**DUSP6**	-4.233	**SUMO2**	2.73	**YWHAZ**	-3.362
MED25	2.232	**PRPS2**	-4.109	**RCE1**	2.715	**WASPIP**	-3.325
DACT3	2.223	**DDX58**	-4.085	**PTPMT1**	2.69	**SPRY2**	-3.196
NUMA1	2.124	**DKK1**	-4.018	**HOXC6**	2.663	**RAD17**	-3.17
DNAJA3	2.113	**SDCBP**	-3.874	**H3F3B**	2.633	**FRMD6**	-3.158
MXD4	2.018	**VCL**	-3.829	**NCOR2**	2.551	**TGFBR2**	-3.154
EIF1	2.017	**CYP2J2**	-3.777	**PLAC8**	2.516	**KLK3**	-3.093
MSX1	1.973	**ITGB1**	-3.766	**EMP1**	2.435	**CD83**	-3.068
ACTN4	1.946	**NFKBIA**	-3.735	**DIXDC1**	2.364	**TOM1L1**	-2.981
HSPA1B	1.939	**E2F5**	-3.714	**YWHAG**	2.3	**AKAP12**	-2.889
CDKN2C	1.935	**OPA1**	-3.536	**S100A4**	2.256	**SPRY1**	-2.87
GPR56	1.925	**CXCL1**	-3.471	**PFDN5**	2.173	**ASPH**	-2.842
NR2F2	1.864	**F2RL1**	-3.358	**H2AFX**	2.155	**TFPI**	-2.787
LGALS7	1.803	**NQO1**	-3.309	**CORO1B**	2.131	**BMPR2**	-2.784
TSC22D3	1.778	**DOCK1**	-3.257	**SERPINH1**	2.094	**DUSP5**	-2.772
MUL1	1.731	**RAF1**	-3.186	**SRF**	2.08	**EIF4G2**	-2.741
COMMD5	1.722	**NRG1**	-3.129	**PIM3**	2.073	**RB1**	-2.707
CDKN2D	1.717	**SEMA4D**	-3.083	**EWSR1**	2.041	**SPRY4**	-2.691
HRASLS	1.714	**VASP**	-3.034	**CBS**	2.022	**PPP2R2C**	-2.691
AGR2	1.713	**CTNNB1**	-3.028	**ACLY**	2.006	**CASP2**	-2.648
RPL26	1.712	**PPP1R13L**	-3.01	**TBP**	2.003	**SMAD3**	-2.627
SH2B3	1.705	**KIF2A**	-2.967	**NDEL1**	1.973	**PNPT1**	-2.597
DAXX	1.703	**CD151**	-2.924	**ISG15**	1.97	**NFE2L2**	-2.573
MTA1	1.691	**BIRC2**	-2.921	**NOP58**	1.961	**ABCC5**	-2.562
CBL	1.683	**CCL20**	-2.862	**TPD52**	1.951	**EIF2AK2**	-2.549
STRA13	1.68	**NASP**	-2.853	**RPS15A**	1.938	**SERINC3**	-2.518
RALBP1	1.655	**SERPINE1**	-2.835	**NACA**	1.927	**CAV1**	-2.515
PEBP1	1.63	**EOMES**	-2.815	**POLR2L**	1.917	**CAPRIN2**	-2.477
CDKN1C	1.62	**PLK2**	-2.805	**NOLC1**	1.909	**RASA1**	-2.476
EI24	1.604	**LCN2**	-2.759	**ATP5G1**	1.893	**IFNGR1**	-2.47
ATPIF1	1.6	**TXNDC5**	-2.702	**ARF1**	1.885	**PIAS3**	-2.463
CD24	1.6	**AFAP1**	-2.7	**LSMD1**	1.868	**RNASEL**	-2.461
ERF	1.59	**ERCC1**	-2.684	**MCM8**	1.851	**TRIB3**	-2.368
NFKBIB	1.581	**ADAR**	-2.639	**DVL2**	1.826	**BHLHB2**	-2.357
ARID1A	1.575	**NBN**	-2.614	**CTSD**	1.819	**CBFB**	-2.357
EMD	1.558	**MAPK9**	-2.609	**LGALS1**	1.802	**HAS3**	-2.356
MNX1	1.554	**YAP1**	-2.596	**NAP1L1**	1.802	**BTG2**	-2.355
PKD1	1.553	**PRKAA1**	-2.582	**SPTAN1**	1.793	**TFPI2**	-2.296
DBI	1.551	**STAM2**	-2.568	**ETS1**	1.787	**ROCK1**	-2.293
LAMA5	1.54	**MCL1**	-2.526	**CREB1**	1.782	**DFNA5**	-2.283
NDUFS3	1.539	**DNM1L**	-2.506	**PIP5K2B**	1.78	**PMP22**	-2.272
GPI	1.538	**IER3**	-2.506	**CSF2RA**	1.777	**KDM3B**	-2.263
UXT	1.536	**SSR1**	-2.498	**VKORC1**	1.777	**APBB2**	-2.252
DNAJB6	1.534	**CD81**	-2.482	**RPS9**	1.755	**PPP2R1B**	-2.252
GADD45A	1.519	**CDC16**	-2.468	**CDC42**	1.731	**NOTCH2**	-2.247
C1QL4	1.509	**PIGN**	-2.447	**DPYSL2**	1.725	**TICAM2**	-2.242

GNE	1.508	**SGK3**	-2.441	**E2F4**	1.721	**CTH**	-2.236
MAFG	1.502	**JAK1**	-2.43	**DYRK1B**	1.716	**G3BP1**	-2.234
IFITM1	1.501	**NOC3L**	-2.425	**RANBP1**	1.715	**CRLF3**	-2.228
		DICER1	-2.424	**PSMA4**	1.711	**NDFIP1**	-2.216
		CCNB1	-2.424	**AHNAK**	1.71	**CASP7**	-2.216
		CCDC6	-2.416	**CRCP**	1.707	**TAX1BP3**	-2.213
		TNF	-2.402	**LEP**	1.704	**ITGA6**	-2.21
		B4GALT6	-2.395	**HS.57079**	1.694	**ROCK2**	-2.194
		ADCY3	-2.394	**S100A6**	1.681	**MERTK**	-2.188
		CD44	-2.382	**HS.535028**	1.67	**UBE2D3**	-2.184
		REL	-2.38	**BASP1**	1.663	**CAT**	-2.164
		ITGB4	-2.377	**MKL1**	1.657	**WDR48**	-2.144
		NR2C2	-2.373	**ILF3**	1.652	**SEC23A**	-2.123
		MAP3K11	-2.354	**ODC1**	1.652	**ERRFI1**	-2.107
		PDE4D	-2.344	**S100A13**	1.649	**LIFR**	-2.1
		EIF2C2	-2.342	**HSPA5**	1.648	**PKP2**	-2.097
		HS.128753	-2.337	**CYBA**	1.645	**RBL2**	-2.096
		CCDC115	-2.337	**GPR177**	1.644	**RNF14**	-2.086
		RAB22A	-2.332	**CCNF**	1.642	**QPCT**	-2.085
		RAN	-2.33	**CTSB**	1.64	**TJP1**	-2.079
		SDC4	-2.323	**IL11**	1.634	**RCHY1**	-2.075
		ELMO2	-2.316	**DIDO1**	1.63	**RNF10**	-2.061
		USP18	-2.311	**VEGFB**	1.627	**ANXA7**	-2.048
		CTTN	-2.309	**PPARBP**	1.613	**JUNB**	-2.031
		ATP7A	-2.299	**ICMT**	1.609	**KIFAP3**	-2.015
		CHUK	-2.299	**ISG20**	1.6	**KRIT1**	-2.004
		RNMT	-2.288	**CDC25B**	1.597	**WTAP**	-1.972
		TRPC1	-2.287	**PPL**	1.587	**B3GNT2**	-1.968
		MAP3K7	-2.285	**BCL2L1**	1.587	**TBK1**	-1.966
		ICAM1	-2.285	**CDK6**	1.581	**CUL2**	-1.949
		LATS2	-2.276	**SHC1**	1.58	**APPL1**	-1.947
		BIRC5	-2.274	**MGAT3**	1.579	**PTPN12**	-1.934
		GFM1	-2.27	**MXD3**	1.575	**S100A11**	-1.932
		CX3CL1	-2.264	**NME3**	1.574	**DAB2**	-1.919
		PPP3CB	-2.249	**ELAVL1**	1.574	**SFN**	-1.916
		TGIF1	-2.236	**SELK**	1.573	**PARG**	-1.915
		MLL3	-2.234	**STX3**	1.571	**ZFP36**	-1.914
		IL13RA1	-2.222	**MCM4**	1.549	**TNFAIP3**	-1.913
		NUP98	-2.219	**DCLRE1A**	1.547	**TUSC2**	-1.907
		MAP2K1	-2.213	**SEC61G**	1.54	**OSBPL2**	-1.907
		FOSL1	-2.21	**FASN**	1.536	**PPM1A**	-1.897
		LIN28B	-2.204	**AES**	1.529	**PLOD2**	-1.894
		CENPF	-2.199	**PTGDS**	1.527	**KLF10**	-1.893
		SOX8	-2.198	**SNX12**	1.527	**MAP3K7IP**	-1.889
		HEYL	-2.175	**SUMO1**	1.523	**CASP3**	-1.884
		CSNK1G3	-2.175	**GLRX**	1.521	**SPRED1**	-1.882
		NFKB1	-2.167	**NSA2**	1.521	**DYRK1A**	-1.877
		CDK2	-2.158	**HES6**	1.52	**SGPL1**	-1.877
		STAT4	-2.128	**MVP**	1.518	**ATG7**	-1.869

		EBI3	-2.121	**PEG10**	1.518	**ELF1**	-1.868
		SLC12A2	-2.114	**CISD1**	1.518	**OAS3**	-1.862
		PDPK1	-2.104	**COX17**	1.514	**FBXO11**	-1.856
		SMO	-2.098	**SLC44A4**	1.514	**MIB1**	-1.849
		ACVR1	-2.095	**SLC25A33**	1.51	**INPPL1**	-1.849
		LBR	-2.09	**EDF1**	1.508	**ASNS**	-1.844
		DDX5	-2.089	**ULK1**	1.501	**PTX3**	-1.843
		SGK1	-2.077	**ABCG1**	1.5	**PCYOX1**	-1.843
		AMACR	-2.07			**PPARA**	-1.841
		TPR	-2.064			**ABLIM1**	-1.833
		UPP1	-2.06			**IRF1**	-1.833
		RIPK1	-2.06			**PPARG**	-1.831
		PIK3R2	-2.06			**DCBLD2**	-1.829
		PIK3CA	-2.054			**TES**	-1.826
		SLC20A1	-2.054			**CHEK2**	-1.824
		FAT4	-2.053			**TRIM24**	-1.803
		CUL4A	-2.051			**SMAD4**	-1.8
		ZNF16	-2.05			**BAT3**	-1.795
		IDE	-2.049			**CTNNBIP1**	-1.794
		PTPN3	-2.044			**VHL**	-1.794
		NCCRP1	-2.027			**E2F7**	-1.786
		CUL1	-2.025			**ULK2**	-1.784
		CDC14B	-2.022			**CCNG2**	-1.781
		CXCL2	-2.021			**NPTX1**	-1.78
		PTHLH	-2.018			**ABTB1**	-1.777
		DSG2	-2.015			**TNFRSF1B**	-1.771
		HS.570988	-2.008			**CYLD**	-1.771
		IDH2	-2.008			**LIPA**	-1.77
		YES1	-2.004			**VPS39**	-1.766
		VGF	-2.002			**P4HA2**	-1.758
		TIMP1	-2.002			**ARID4B**	-1.754
		SPTBN1	-2.002			**IQGAP1**	-1.751
		PHIP	-1.999			**NME6**	-1.746
		MKNK1	-1.998			**USP16**	-1.741
		S1PR3	-1.994			**TRIM21**	-1.739
		DDX21	-1.992			**JARID2**	-1.736
		USP47	-1.99			**GEM**	-1.736
		GAB2	-1.989			**VAMP7**	-1.733
		DUSP10	-1.986			**PARP10**	-1.723
		UBE2A	-1.985			**FAM188A**	-1.722
		HSPA4	-1.984			**IL28A**	-1.722
		NAE1	-1.982			**DUSP1**	-1.72
		HNRPK	-1.981			**PAK1IP1**	-1.72
		ARFGAP3	-1.98			**CD40**	-1.717
		MINA	-1.976			**TANK**	-1.717
		ADAM17	-1.975			**AKTIP**	-1.717
		CIAO1	-1.972			**ARID4A**	-1.715
		SEMA4C	-1.971			**CYP2S1**	-1.712
		PAPSS2	-1.967			**TNFRSF1A**	-1.708

		CLN3	-1.966			**DNER**	-1.708
		E2F3	-1.964			**HS.371609**	-1.7
		NFYA	-1.962			**PPP2R5C**	-1.695
		ARFGEF1	-1.959			**SRM**	-1.694
		CYR61	-1.957			**EPHX2**	-1.69
		SERTAD1	-1.953			**RAPGEF2**	-1.688
		TRIM39	-1.95			**HIAT1**	-1.688
		KIAA1429	-1.944			**EFNA1**	-1.684
		TNFAIP8	-1.942			**OBFC2A**	-1.68
		PTPRA	-1.941			**CDKN1A**	-1.678
		SUV420H1	-1.94			**AXIN1**	-1.676
		RPS6KA3	-1.939			**SESN1**	-1.675
		CD164	-1.937			**SERPINA3**	-1.675
		NCK1	-1.935			**CBLB**	-1.673
		LAP3	-1.934			**DDX3X**	-1.669
		PRKAR1A	-1.933			**PROS1**	-1.668
		TIRAP	-1.933			**PPP2CA**	-1.667
		PIK3C2A	-1.931			**TLR6**	-1.666
		ZMYM2	-1.931			**TP53BP2**	-1.659
		GCH1	-1.93			**CASK**	-1.659
		UGCG	-1.928			**ENPP1**	-1.657
		OPTN	-1.926			**ITPR1**	-1.653
		CSNK2A1	-1.924			**RHOB**	-1.651
		ITGAV	-1.924			**VLDLR**	-1.648
		ACTB	-1.921			**PHF14**	-1.647
		P4HA1	-1.911			**KEAP1**	-1.644
		CITED2	-1.909			**STX2**	-1.641
		PRMT6	-1.908			**RANBP9**	-1.641
		KPNA2	-1.905			**PXMP3**	-1.639
		IMPACT	-1.904			**MORC3**	-1.622
		EXOC5	-1.899			**GABPB2**	-1.62
		AMD1	-1.898			**MINPP1**	-1.62
		GAD1	-1.897			**DYNC1H1**	-1.617
		NPC1	-1.897			**TOP1**	-1.615
		SPAST	-1.896			**KLF6**	-1.611
		ZNF259	-1.893			**ADIPOR1**	-1.603
		PRKCA	-1.889			**TAP1**	-1.602
		DDX17	-1.887			**CAPRIN1**	-1.6
		CCPG1	-1.887			**B3GNT5**	-1.599
		RAB5A	-1.883			**LAT2**	-1.592
		FOXQ1	-1.88			**SAV1**	-1.59
		MAPK1	-1.879			**AATF**	-1.585
		FNDC3B	-1.879			**TMEM127**	-1.584
		MYD88	-1.878			**RNF4**	-1.58
		KIAA0261	-1.876			**UBIAD1**	-1.579
		TFDP1	-1.875			**FOXF2**	-1.578
		ITGA2	-1.873			**RND3**	-1.577
		ARHGAP1	-1.873			**CTNNA1**	-1.576
		ACTL6A	-1.873			**PARK2**	-1.575

		JUN	-1.871			**SMYD2**	-1.574
		XRCC5	-1.856			**MXD1**	-1.572
		STK38L	-1.848			**CSNK1A1**	-1.571
		ADAM9	-1.842			**ABI1**	-1.569
		FAS	-1.837			**RHOH**	-1.569
		TLN1	-1.837			**CTCF**	-1.566
		SS18	-1.833			**CSE1L**	-1.566
		EML4	-1.831			**KIF23**	-1.565
		KRAS	-1.824			**IGFBP4**	-1.559
		PDGFC	-1.82			**IL28B**	-1.558
		PPP1CA	-1.818			**FKTN**	-1.551
		SHMT1	-1.816			**CDK8**	-1.549
		DLGAP5	-1.813			**BACH2**	-1.546
		AKIRIN1	-1.812			**TAF6**	-1.546
		DDAH1	-1.81			**STRN**	-1.541
		NAMPT	-1.808			**ABCC1**	-1.541
		KIF20B	-1.807			**PTPN2**	-1.54
		RBM3	-1.798			**TOPBP1**	-1.534
		GLDC	-1.797			**RNF111**	-1.534
		VEGFC	-1.796			**H2AFY**	-1.534
		MIR21	-1.795			**IFT57**	-1.531
		RECQL	-1.794			**RB1CC1**	-1.523
		API5	-1.794			**PBRM1**	-1.523
		STK39	-1.793			**TCF12**	-1.522
		HS.334831	-1.789			**HNRNPL**	-1.522
		CBX2	-1.785			**HES1**	-1.522
		BCAT1	-1.782			**IVNS1ABP**	-1.519
		LEPR	-1.779			**EMILIN2**	-1.518
		LARP1	-1.778			**CDC2L1**	-1.516
		RGPD8	-1.776			**IFIT3**	-1.515
		RFC1	-1.775			**RBM5**	-1.514
		BMPR1A	-1.775			**ATF2**	-1.513
		ADAM10	-1.774			**FBLN1**	-1.513
		CDC2L6	-1.774			**KRT19**	-1.511
		ZBED1	-1.774			**SOX17**	-1.511
		CD46	-1.773			**MAPT**	-1.51
		ASCC3	-1.769			**CTBP2**	-1.508
		ALCAM	-1.768			**DNM2**	-1.506
		WRN	-1.767			**SOCS6**	-1.506
		ABI2	-1.765			**DCUN1D3**	-1.504
		CDC2	-1.761			**ATG5**	-1.503
		AHR	-1.759			**MCRS1**	-1.502
		POT1	-1.759				
		VANGL1	-1.758				
		ATMIN	-1.758				
		EXTL3	-1.758				
		BPNT1	-1.756				
		MAP2K5	-1.755				
		APP	-1.751				

		RCAN1	-1.75				
		PHCA	-1.75				
		STAT5B	-1.749				
		WWTR1	-1.746				
		GCLC	-1.744				
		LTB	-1.743				
		FBXW11	-1.737				
		NFKB2	-1.737				
		SMARCB1	-1.734				
		PLD1	-1.734				
		CLIP1	-1.732				
		MAP4K4	-1.729				
		FER	-1.721				
		USP22	-1.721				
		PDE5A	-1.718				
		ULBP1	-1.717				
		GOLPH3	-1.717				
		CERK	-1.717				
		PSME3	-1.716				
		FKBP1B	-1.715				
		CCNDBP1	-1.714				
		TRIO	-1.712				
		PTGER4	-1.712				
		USO1	-1.712				
		HMGA1	-1.711				
		FER1L3	-1.71				
		F2R	-1.706				
		LSM1	-1.704				
		NCOA2	-1.703				
		HMGCR	-1.701				
		KATNA1	-1.701				
		BCL2A1	-1.7				
		PLCE1	-1.7				
		RAB28	-1.699				
		AURKA	-1.697				
		FGFR4	-1.693				
		KIF18A	-1.688				
		MCMDC1	-1.686				
		TOP1MT	-1.686				
		ERBB2	-1.683				
		PTPN13	-1.681				
		BIRC6	-1.68				
		SLC24A6	-1.677				
		PLCG2	-1.676				
		RIMS3	-1.675				
		ERO1LB	-1.673				
		KIAA0776	-1.673				
		EXT1	-1.671				
		HAS2	-1.67				

		ZEB1	-1.67				
		TP53BP1	-1.667				
		ZNF451	-1.667				
		RNF20	-1.664				
		ANGPTL4	-1.663				
		RALA	-1.663				
		RFC3	-1.663				
		EGFR	-1.661				
		EPS8	-1.659				
		MSH3	-1.657				
		TAF9L	-1.657				
		CALCRL	-1.656				
		GPR3	-1.652				
		GCNT2	-1.646				
		PAK2	-1.645				
		FIS1	-1.645				
		MALT1	-1.643				
		WWC1	-1.642				
		FKBP1A	-1.64				
		GNAQ	-1.638				
		NEDD4L	-1.637				
		PPIA	-1.636				
		PIK3CB	-1.636				
		KIF11	-1.636				
		BARD1	-1.635				
		OSBPL1A	-1.63				
		NFATC3	-1.629				
		RBCK1	-1.626				
		CD59	-1.625				
		RIPK2	-1.624				
		MTDH	-1.623				
		RAD50	-1.619				
		ANXA2	-1.619				
		DLL1	-1.615				
		ACVR2A	-1.614				
		C1GALT1C1	-1.614				
		PLS3	-1.608				
		CDC2L5	-1.607				
		CNOT2	-1.607				
		FNTB	-1.606				
		E2F6	-1.606				
		XRCC4	-1.606				
		FGFR1OP	-1.603				
		FYN	-1.601				
		MLL5	-1.601				
		ETV4	-1.6				
		CCT2	-1.597				
		TTLL4	-1.596				
		ECT2	-1.593				

		UBR5	-1.592				
		EPAS1	-1.59				
		PRCC	-1.59				
		PRKD3	-1.59				
		STT3B	-1.59				
		NOD2	-1.586				
		IFNAR2	-1.586				
		CAPN2	-1.585				
		CAMK2D	-1.585				
		EIF2C3	-1.584				
		STK24	-1.583				
		CCNA2	-1.583				
		TRADD	-1.582				
		RAB1A	-1.58				
		MAP3K7IP2	-1.579				
		PIK3R1	-1.578				
		GPR172B	-1.578				
		STAG1	-1.578				
		RPRD1B	-1.578				
		PIK3C2B	-1.576				
		MAPK13	-1.572				
		DNAJA2	-1.572				
		SLC2A1	-1.571				
		SOCS2	-1.568				
		RHOQ	-1.568				
		JAK2	-1.568				
		NUDCD3	-1.564				
		THOC1	-1.564				
		MAP3K4	-1.563				
		TESK1	-1.562				
		STAT6	-1.561				
		TYMS	-1.56				
		MSRA	-1.558				
		EIF2B2	-1.557				
		C1GALT1	-1.557				
		SFRS5	-1.556				
		MUC1	-1.556				
		RECK	-1.555				
		GJC1	-1.552				
		ARHGEF6	-1.551				
		ARNTL	-1.55				
		RBBP9	-1.55				
		STEAP2	-1.547				
		HGF	-1.545				
		KDM5B	-1.543				
		AKR1C3	-1.543				
		WASF2	-1.54				
		TBX1	-1.54				
		SMC3	-1.54				

		BLM	-1.538				
		MMP25	-1.537				
		TICAM1	-1.536				
		CISD2	-1.533				
		CD2AP	-1.532				
		TDG	-1.531				
		TADA3	-1.529				
		RNF144B	-1.528				
		NFS1	-1.525				
		SLC30A6	-1.524				
		AKAP13	-1.523				
		HIP1	-1.52				
		BUB3	-1.519				
		NRIP1	-1.519				
		ARRB2	-1.519				
		YEATS4	-1.519				
		CASP6	-1.519				
		RCL1	-1.519				
		FRAP1	-1.518				
		AKT2	-1.516				
		ACSL4	-1.515				
		CXCR4	-1.515				
		PFKFB3	-1.514				
		PALLD	-1.512				
		AGGF1	-1.507				
		EZH2	-1.503				
		MAP2K4	-1.503				
		ST5	-1.502				
		GLB1	-1.502				
		ASPM	-1.501				
		STK38	-1.501				

B.

Expected				**Inversely related**			
increased cell death				**decreased cell death**			
up-genes (66)	fold change	**down genes (393)**	fold change	**up-genes (94)**	fold change	**down genes (288)**	fold change
TXNIP	5.257	**RRM2**	-7.011	**NUAK1**	3.768	**EGR1**	-15.086
SREBF1	4.275	**IL8**	-6.204	**DEPDC6**	3.277	**RAC1**	-8.138
SET	3.643	**BIRC3**	-6.182	**TFF3**	3.153	**CD69**	-7.186
HMGB2	2.987	**TRIB1**	-4.97	**LRPAP1**	2.864	**FOS**	-5.573
HMGB1	2.955	**CTGF**	-4.591	**SIP1**	2.815	**MX1**	-5.51
SMARCC1	2.9	**CAST**	-4.411	**STX8**	2.777	**GULP1**	-4.962
ITGB3BP	2.747	**PLAU**	-4.394	**PTPMT1**	2.69	**B4GALT5**	-4.263
NCOR2	2.551	**CDCP1**	-4	**HOXC6**	2.663	**DUSP6**	-4.233
EMP1	2.435	**PPP2R2A**	-3.901	**PLAC8**	2.516	**DDX58**	-4.085

FAM162A	2.329	**WEE1**	-3.845	**DAG1**	2.452	**STAT1**	-4.047
MYH9	2.289	**VCL**	-3.829	**DDIT4**	2.389	**TNFRSF21**	-4.036
H2AFX	2.155	**CYP2J2**	-3.777	**FBXO2**	2.378	**OLR1**	-4.027
NUMA1	2.124	**NFKBIA**	-3.735	**YWHAG**	2.3	**DKK1**	-4.018
DNAJA3	2.113	**OPA1**	-3.536	**S100A4**	2.256	**NFKBIZ**	-3.78
DPM3	2.007	**SP3**	-3.521	**ACO2**	2.249	**ITGB1**	-3.766
ACLY	2.006	**CXCL1**	-3.471	**SIGIRR**	2.242	**STEAP3**	-3.66
BOK	2.003	**SRXN1**	-3.422	**TAOK3**	2.196	**PHLDA1**	-3.657
ISG15	1.97	**EGR2**	-3.392	**HSPBP1**	2.146	**IFIH1**	-3.606
CDKN2C	1.935	**SIAH2**	-3.389	**RGS10**	2.127	**PRMT2**	-3.414
EGLN2	1.882	**YWHAZ**	-3.362	**WDR4**	2.114	**F2RL1**	-3.358
IL17RD	1.873	**SLC4A7**	-3.353	**SERPINH1**	2.094	**WASPIP**	-3.325
LAMP1	1.843	**STK40**	-3.272	**SRF**	2.08	**NQO1**	-3.309
IL10	1.834	**SPRY2**	-3.196	**MEF2D**	2.078	**DOCK1**	-3.257
HS1BP3	1.825	**RAF1**	-3.186	**PIM3**	2.073	**FRMD6**	-3.158
TGFBI	1.808	**TGFBR2**	-3.154	**CAMK2G**	2.064	**NRG1**	-3.129
LGALS7	1.803	**VPS4B**	-3.147	**EWSR1**	2.041	**PERP**	-2.924
LGALS1	1.802	**NEK6**	-3.142	**INTS1**	2.028	**TFPI**	-2.787
ETS1	1.787	**LGMN**	-3.136	**CBS**	2.022	**ANTXR2**	-2.773
RASSF6	1.787	**KLK3**	-3.093	**TBP**	2.003	**CTSL1**	-2.763
HIST1H1C	1.781	**RRM2B**	-3.053	**MSX1**	1.973	**LCN2**	-2.759
PRELID1	1.781	**VASP**	-3.034	**NDEL1**	1.973	**IL1R1**	-2.75
CSF2RA	1.777	**CTNNB1**	-3.028	**TPD52**	1.951	**AFAP1**	-2.7
PTRH2	1.754	**PPP1R13L**	-3.01	**HSPA1B**	1.939	**CASP2**	-2.648
FAU	1.737	**GLUD1**	-2.966	**PINK1**	1.915	**GPR37**	-2.639
CDC42	1.731	**CD151**	-2.924	**NOLC1**	1.909	**SMAD3**	-2.627
PRPS1	1.727	**BIRC2**	-2.921	**LSMD1**	1.868	**MAPK9**	-2.609
HRASLS	1.714	**AKAP12**	-2.889	**DYNLL1**	1.854	**PNPT1**	-2.597
AGR2	1.713	**SERPINE1**	-2.835	**TUB**	1.812	**STAM2**	-2.568
DAXX	1.703	**EOMES**	-2.815	**DMC1**	1.803	**EIF2AK2**	-2.549
S100A6	1.681	**PLK2**	-2.805	**CREB1**	1.782	**SERINC3**	-2.518
ENDOG	1.666	**EIF4G2**	-2.741	**UBQLN1**	1.778	**ATP2A2**	-2.512
CYBA	1.645	**VAMP3**	-2.717	**TSC22D3**	1.778	**DNM1L**	-2.506
CTSB	1.64	**RB1**	-2.707	**NKX6-2**	1.774	**IER3**	-2.506
ADRM1	1.633	**TXNDC5**	-2.702	**TRIAP1**	1.743	**CAPRIN2**	-2.477
TMEM107	1.631	**ERCC1**	-2.684	**E2F4**	1.721	**RASA1**	-2.476
PEBP1	1.63	**ADAR**	-2.639	**CDKN2D**	1.717	**IFNGR1**	-2.47
CDKN1C	1.62	**NBN**	-2.614	**NUF2**	1.717	**PIAS3**	-2.463
PPARBP	1.613	**YAP1**	-2.596	**LEP**	1.704	**RNASEL**	-2.461
CD24	1.6	**PRKAA1**	-2.582	**CALM3**	1.695	**JAK1**	-2.43
PRR7	1.586	**NFE2L2**	-2.573	**CBL**	1.683	**CCNB1**	-2.424
NFKBIB	1.581	**AADACL1**	-2.568	**PI4KB**	1.667	**CCDC6**	-2.416
CDK6	1.581	**ABCC5**	-2.562	**CBX4**	1.662	**TNF**	-2.402
SHC1	1.58	**MCL1**	-2.526	**MKL1**	1.657	**SCP2**	-2.396
NME3	1.574	**CAV1**	-2.515	**ILF3**	1.652	**TRIB3**	-2.368
UBE2L3	1.549	**ERC1**	-2.476	**ODC1**	1.652	**ZFYVE16**	-2.362
BRMS1	1.548	**TPM3**	-2.476	**STXBP1**	1.648	**HAS3**	-2.356

MRPL41	1.545	**PIGA**	-2.451	**HSPA5**	1.648	**MAP3K11**	-2.354
LAMA5	1.54	**SGK3**	-2.441	**TNNI3**	1.639	**PARP14**	-2.35
AES	1.529	**ANXA4**	-2.427	**IL11**	1.634	**LGALS8**	-2.334
PTGDS	1.527	**DICER1**	-2.424	**DIDO1**	1.63	**RAB22A**	-2.332
PRAF2	1.527	**ABCE1**	-2.389	**LMO4**	1.627	**OAS1**	-2.332
SUMO1	1.523	**MBTPS1**	-2.388	**VEGFB**	1.627	**SDC4**	-2.323
MAP3K12	1.522	**CD44**	-2.382	**ICMT**	1.609	**SLC25A24**	-2.323
GADD45A	1.519	**REL**	-2.38	**PLEC1**	1.606	**TFPI2**	-2.296
MVP	1.518	**ITGB4**	-2.377	**EI24**	1.604	**ICAM1**	-2.285
MT1F	1.503	**NR2C2**	-2.373	**OSCAR**	1.602	**LATS2**	-2.276
		HIPK3	-2.362	**ERF**	1.59	**ZFAND5**	-2.273
		BHLHB2	-2.357	**BCL2L1**	1.587	**PMP22**	-2.272
		CBFB	-2.357	**MGAT3**	1.579	**CX3CL1**	-2.264
		BTG2	-2.355	**YWHAE**	1.576	**USP12**	-2.251
		ADH5	-2.353	**MXD3**	1.575	**NOTCH2**	-2.247
		SELE	-2.349	**ARID1A**	1.575	**TICAM2**	-2.242
		CES1	-2.348	**ELAVL1**	1.574	**CTH**	-2.236
		PDE4D	-2.344	**GADD45GIP1**	1.563	**ICAM3**	-2.236
		EIF2C2	-2.342	**EMD**	1.558	**TJP2**	-2.228
		HS.128753	-2.337	**TAF10**	1.553	**CASP7**	-2.216
		CYB5A	-2.321	**PKD1**	1.553	**XPR1**	-2.216
		USP18	-2.311	**BCL2L12**	1.552	**FOSL1**	-2.21
		CTTN	-2.309	**SEC61G**	1.54	**ITGA6**	-2.21
		ATP7A	-2.299	**CALR**	1.539	**ROCK2**	-2.194
		CHUK	-2.299	**GPI**	1.538	**MAP2K3**	-2.193
		TRPC1	-2.287	**WFS1**	1.537	**BID**	-2.178
		MAP3K7	-2.285	**UXT**	1.536	**PRKDC**	-2.171
		SMARCA5	-2.285	**FASN**	1.536	**ELF4**	-2.159
		SRI	-2.279	**DNAJB6**	1.534	**CDK2**	-2.158
		BIRC5	-2.274	**HBXIP**	1.531	**CCDC109A**	-2.149
		IPPK	-2.263	**EBAG9**	1.53	**SP110**	-2.147
		PPP2R1B	-2.252	**MAPKAP1**	1.529	**WDR48**	-2.144
		PPP3CB	-2.249	**HSPE1**	1.521	**STAT4**	-2.128
		HSD17B4	-2.229	**PEG10**	1.518	**ATG4A**	-2.095
		MTM1	-2.229	**APRT**	1.514	**DDX5**	-2.089
		MAP2K1	-2.213	**GPX4**	1.504	**CKAP2**	-2.069
		CENPF	-2.199	**MAFG**	1.502	**RIPK1**	-2.06
		SOX8	-2.198	**ABCG1**	1.5	**PIK3R2**	-2.06
		RELB	-2.197			**IDE**	-2.049
		HADHA	-2.197			**NSMAF**	-2.038
		IFI6	-2.195			**TMEM158**	-2.027
		MERTK	-2.188			**SESN2**	-2.019
		ZNF184	-2.177			**DSG2**	-2.015
		HEYL	-2.175			**SENP2**	-2.008
		SERP1	-2.168			**YES1**	-2.004
		NFKB1	-2.167			**VGF**	-2.002
		CAT	-2.164			**SPTBN1**	-2.002

		HS.532698	-2.162			**HMOX2**	-1.996
		MSRB3	-2.161			**ZAK**	-1.993
		PDE4B	-2.127			**NAE1**	-1.982
		SLC12A2	-2.114			**SLC25A38**	-1.979
		PDPK1	-2.104			**WTAP**	-1.972
		RABGGTA	-2.103			**E2F3**	-1.964
		SMO	-2.098			**SNAP25**	-1.957
		RBL2	-2.096			**TIA1**	-1.955
		PAFAH2	-2.093			**APPL1**	-1.947
		DSP	-2.088			**PTPRA**	-1.941
		SGK1	-2.077			**OMA1**	-1.939
		CCNI	-2.077			**NCK1**	-1.935
		AMACR	-2.07			**PRKAR1A**	-1.933
		FIGNL1	-2.066			**TIRAP**	-1.933
		TPR	-2.064			**GCH1**	-1.93
		PIK3CA	-2.054			**PPP2R5A**	-1.925
		SLC20A1	-2.054			**CSNK2A1**	-1.924
		FAT4	-2.053			**TBCCD1**	-1.922
		ZNF16	-2.05			**DAB2**	-1.919
		ANXA7	-2.048			**PARG**	-1.915
		HS.24119	-2.037			**ZFP36**	-1.914
		UBR2	-2.032			**PDCD6IP**	-1.899
		JUNB	-2.031			**PPM1A**	-1.897
		CUL1	-2.025			**CASP3**	-1.884
		PON2	-2.023			**ZMYND11**	-1.88
		CXCL2	-2.021			**MYD88**	-1.878
		PTHLH	-2.018			**SGPL1**	-1.877
		KIFAP3	-2.015			**TFDP1**	-1.875
		GLO1	-2.01			**ACTL6A**	-1.873
		HS.570988	-2.008			**MAP3K8**	-1.871
		IDH2	-2.008			**JUN**	-1.871
		TIMP1	-2.002			**ATG7**	-1.869
		PHIP	-1.999			**OAS3**	-1.862
		MKNK1	-1.998			**RFK**	-1.856
		S1PR3	-1.994			**WWC3**	-1.855
		NSF	-1.992			**MIB1**	-1.849
		USP47	-1.99			**PSEN2**	-1.848
		GAB2	-1.989			**QKI**	-1.845
		DUSP10	-1.986			**TLR1**	-1.84
		HSPA4	-1.984			**TFDP2**	-1.84
		HNRPK	-1.981			**FAS**	-1.837
		ADAM17	-1.975			**IRF1**	-1.833
		TBK1	-1.966			**THAP1**	-1.833
		TOP2B	-1.966			**ANKRD1**	-1.831
		CLN3	-1.966			**PPARG**	-1.831
		TAX1BP1	-1.963			**KRAS**	-1.824
		NFYA	-1.962			**CHEK2**	-1.824

		CYR61	-1.957			**PPP1CA**	-1.818
		SCARB2	-1.95			**ATP1A1**	-1.807
		TRIM39	-1.95			**SMAD4**	-1.8
		CUL2	-1.949			**FAF1**	-1.798
		TNFAIP8	-1.942			**BAT3**	-1.795
		RPS6KA3	-1.939			**VHL**	-1.794
		SOX21	-1.939			**TSC22D2**	-1.791
		CD164	-1.937			**ULK2**	-1.784
		S100A11	-1.932			**GSR**	-1.78
		ZMYM2	-1.931			**NPTX1**	-1.78
		PPP1R2	-1.93			**GMCL1**	-1.773
		UGCG	-1.928			**TNFRSF1B**	-1.771
		OPTN	-1.926			**CYLD**	-1.771
		ITGAV	-1.924			**LIPA**	-1.77
		BBS2	-1.918			**CCNC**	-1.763
		SFN	-1.916			**CDC2**	-1.761
		DCAF7	-1.914			**MAP2K5**	-1.755
		APIP	-1.914			**APP**	-1.751
		TNFAIP3	-1.913			**XPC**	-1.75
		CITED2	-1.909			**STAP2**	-1.744
		KPNA2	-1.905			**LTB**	-1.743
		LRP8	-1.898			**TRIM21**	-1.739
		NPC1	-1.897			**UACA**	-1.732
		ZNF259	-1.893			**SH3RF1**	-1.732
		PRKCA	-1.889			**MAP4K4**	-1.729
		THOC6	-1.889			**PKN2**	-1.727
		HDAC9	-1.882			**PDE5A**	-1.718
		MAPK1	-1.879			**ULBP1**	-1.717
		KIAA0261	-1.876			**CD40**	-1.717
		ITGA2	-1.873			**PSME3**	-1.716
		ARHGAP1	-1.873			**PTGER4**	-1.712
		DEK	-1.87			**SLC47A1**	-1.71
		XRCC5	-1.856			**SDC1**	-1.709
		ATP2B1	-1.852			**ATG16L1**	-1.709
		ASNS	-1.844			**TNFRSF1A**	-1.708
		PPARA	-1.841			**F2R**	-1.706
		VPS13A	-1.84			**ATXN3**	-1.705
		SERPINI1	-1.833			**HS.371609**	-1.7
		VPS41	-1.823			**HS.4988**	-1.698
		PDGFC	-1.82			**ALDH1A3**	-1.697
		GRB10	-1.82			**FGFR4**	-1.693
		KIF14	-1.816			**MBD1**	-1.689
		ANXA5	-1.814			**M6PR**	-1.688
		SETX	-1.814			**RAPGEF2**	-1.688
		SPG7	-1.812			**PTPN13**	-1.681
		NAMPT	-1.808			**AXIN1**	-1.676
		TRIM24	-1.803			**DDX3X**	-1.669

		RBM3	-1.798			**APOBEC3B**	-1.667
		VEGFC	-1.796			**PPP2CA**	-1.667
		BCL2L13	-1.795			**GFPT1**	-1.666
		MIR21	-1.795			**TP53BP2**	-1.659
		RECQL	-1.794			**TNFRSF10A**	-1.657
		CTNNBIP1	-1.794			**ITPR1**	-1.653
		API5	-1.794			**RHOB**	-1.651
		HS.334831	-1.789			**PAK2**	-1.645
		DHX9	-1.788			**FIS1**	-1.645
		E2F7	-1.786			**PRKRA**	-1.645
		SON	-1.782			**KEAP1**	-1.644
		LEPR	-1.779			**MALT1**	-1.643
		NPHS1	-1.777			**EBF4**	-1.643
		RGPD8	-1.776			**RANBP9**	-1.641
		RFC1	-1.775			**FKBP1A**	-1.64
		CD46	-1.773			**GNAQ**	-1.638
		LASS6	-1.769			**SEC23B**	-1.637
		ALCAM	-1.768			**BARD1**	-1.635
		AHR	-1.759			**NLRX1**	-1.631
		POT1	-1.759			**DEPDC1**	-1.63
		ATMIN	-1.758			**MIR302C**	-1.629
		IRF2	-1.752			**ARHGEF7**	-1.624
		ERCC5	-1.751			**RIPK2**	-1.624
		RCAN1	-1.75			**SUB1**	-1.621
		STAT5B	-1.749			**DDX20**	-1.62
		SLC40A1	-1.749			**SRPX**	-1.619
		PRKAA2	-1.745			**TOP1**	-1.615
		GCLC	-1.744			**FYN**	-1.601
		SLC9A4	-1.743			**ECT2**	-1.593
		SNX7	-1.741			**SAV1**	-1.59
		UBE2V1	-1.737			**IFNAR2**	-1.586
		NFKB2	-1.737			**CAPN2**	-1.585
		GEM	-1.736			**STK24**	-1.583
		FBXL5	-1.734			**HIP2**	-1.583
		PLD1	-1.734			**TRADD**	-1.582
		PARP16	-1.732			**ATP6AP2**	-1.582
		CCT6A	-1.729			**RAB1A**	-1.58
		LASS2	-1.724			**MAL**	-1.579
		IL28A	-1.722			**FOXF2**	-1.578
		FER	-1.721			**STAG1**	-1.578
		USP22	-1.721			**MELK**	-1.578
		DUSP1	-1.72			**CTNNA1**	-1.576
		PLS1	-1.718			**MAPK13**	-1.572
		DPYD	-1.718			**RHOH**	-1.569
		TANK	-1.717			**CSE1L**	-1.566
		CERK	-1.717			**THOC1**	-1.564
		RNF19A	-1.716			**ARL8B**	-1.562

		SQSTM1	-1.716			**ING3**	-1.561
		XPO1	-1.714			**CCNT1**	-1.558
		NEK1	-1.714			**SPATA2**	-1.558
		HMGA1	-1.711			**RECK**	-1.555
		TCERG1	-1.706			**KLRF1**	-1.555
		TMBIM6	-1.703			**TRIM13**	-1.554
		NCOA2	-1.703			**SH2D4A**	-1.553
		HMGCR	-1.701			**ARHGEF6**	-1.551
		BCL2A1	-1.7			**SIK1**	-1.547
		PSMB8	-1.699			**BACH2**	-1.546
		RAB28	-1.699			**TAF6**	-1.546
		AURKA	-1.697			**AKR1C3**	-1.543
		PPP2R5C	-1.695			**PTPN2**	-1.54
		NDST1	-1.695			**BLM**	-1.538
		EFNA1	-1.684			**TICAM1**	-1.536
		ERBB2	-1.683			**ARHGEF3**	-1.532
		GPHN	-1.681			**IFT57**	-1.531
		OBFC2A	-1.68			**RNF144B**	-1.528
		BIRC6	-1.68			**DEDD**	-1.524
		CDKN1A	-1.678			**TCF12**	-1.522
		PLCG2	-1.676			**HES1**	-1.522
		FAM134B	-1.676			**SHQ1**	-1.522
		SERPINA3	-1.675			**TRMT11**	-1.521
		CBLB	-1.673			**HIP1**	-1.52
		PEX11B	-1.672			**CASP6**	-1.519
		HAS2	-1.67			**EMILIN2**	-1.518
		ZEB1	-1.67			**CXCR4**	-1.515
		PROS1	-1.668			**AIFM1**	-1.514
		TP53BP1	-1.667			**RBM5**	-1.514
		TLR6	-1.666			**ATF2**	-1.513
		PDE9A	-1.666			**MAPT**	-1.51
		VAC14	-1.664			**MCOLN3**	-1.51
		ANGPTL4	-1.663			**LASS5**	-1.51
		AVEN	-1.663			**IL17RA**	-1.508
		PRKAG1	-1.661			**DNM2**	-1.506
		EGFR	-1.661			**REPS2**	-1.506
		MSH3	-1.657			**DCUN1D3**	-1.504
		TAF9L	-1.657			**SPAG5**	-1.503
		CALCRL	-1.656			**ZNF274**	-1.503
		ST3GAL1	-1.656			**ATG5**	-1.503
		FSTL1	-1.654			**MAP2K4**	-1.503
		BRE	-1.651			**CCBL1**	-1.502
		HLA-F	-1.647			**ADRB1**	-1.501
		PPIA	-1.636			**STK38**	-1.501
		PIK3CB	-1.636				
		KIF11	-1.636				
		CHMP2A	-1.634				

		GLIS3	-1.628				
		RBCK1	-1.626				
		UBA3	-1.625				
		CD59	-1.625				
		FANCL	-1.625				
		MTDH	-1.623				
		ZFP82	-1.623				
		IFNGR2	-1.62				
		RAD50	-1.619				
		ANXA2	-1.619				
		SEPN1	-1.619				
		DYNC1H1	-1.617				
		IREB2	-1.616				
		DLL1	-1.615				
		KLF6	-1.611				
		CPEB4	-1.611				
		RHBDD1	-1.61				
		EPB41	-1.608				
		CNOT2	-1.607				
		E2F6	-1.606				
		XRCC4	-1.606				
		BAZ1A	-1.605				
		CAPRIN1	-1.6				
		ATP2B4	-1.599				
		HEXB	-1.594				
		PCID2	-1.593				
		NPAS2	-1.592				
		EXOC2	-1.592				
		CCDC47	-1.592				
		CKAP5	-1.592				
		EPAS1	-1.59				
		PRCC	-1.59				
		PRKD3	-1.59				
		FBXO5	-1.587				
		SFRS2B	-1.587				
		AATF	-1.585				
		EIF2C3	-1.584				
		MAN2A1	-1.582				
		DCTN1	-1.581				
		RNF4	-1.58				
		MAP3K7IP2	-1.579				
		PIK3R1	-1.578				
		RND3	-1.577				
		PARK2	-1.575				
		GPN1	-1.574				
		MXD1	-1.572				
		SLC2A1	-1.571				

		PAF1	-1.57				
		SOCS2	-1.568				
		JAK2	-1.568				
		CTCF	-1.566				
		NUDCD3	-1.564				
		MAP3K4	-1.563				
		STAT6	-1.561				
		SLC29A2	-1.56				
		TYMS	-1.56				
		IGFBP4	-1.559				
		MUC1	-1.556				
		LIN7C	-1.555				
		GJC1	-1.552				
		CDK8	-1.549				
		CERKL	-1.549				
		MYO6	-1.548				
		MCPH1	-1.547				
		SPINT1	-1.545				
		HGF	-1.545				
		ADA	-1.544				
		RRAS2	-1.544				
		TBC1D15	-1.543				
		ABCC1	-1.541				
		TOPBP1	-1.534				
		CISD2	-1.533				
		PPID	-1.532				
		MTMR2	-1.532				
		CD2AP	-1.532				
		ATP11C	-1.532				
		DPP8	-1.526				
		TMX1	-1.524				
		RB1CC1	-1.523				
		ARRB2	-1.519				
		IVNS1ABP	-1.519				
		YEATS4	-1.519				
		FRAP1	-1.518				
		AKT2	-1.516				
		ACSL4	-1.515				
		IFIT3	-1.515				
		PLTP	-1.515				
		FBLN1	-1.513				
		PALLD	-1.512				
		SORBS2	-1.512				
		KRT19	-1.511				
		CTBP2	-1.508				
		WASF1	-1.507				
		AGGF1	-1.507				

		TXNRD1	-1.506				
		ATP2C1	-1.506				
		PCNA	-1.505				
		EZH2	-1.503				
		GSTA2	-1.503				
		EFEMP1	-1.502				
		MCRS1	-1.502				

C.

Expected				**Inversely related**			
decreased migration				**increased migration**			
up-genes (7)	fold change	**down genes (122)**	fold change	**up-genes (22)**	fold change	**down genes (23)**	fold change
MYH9	2.289	**EGR1**	-15.086	**C1QBP**	3.473	**RNF144**	-4.078
DNAJA3	2.113	**RAC1**	-8.138	**DPAGT1**	2.397	**STAT1**	-4.047
DPYSL2	1.725	**IL8**	-6.204	**S100A4**	2.256	**ARRDC3**	-3.535
CMTM8	1.706	**CTGF**	-4.591	**ACTN4**	1.946	**LAMC2**	-3.497
BRMS1	1.548	**PLAU**	-4.394	**ARF1**	1.885	**SPRY2**	-3.196
LAMA5	1.54	**DDX58**	-4.085	**DVL2**	1.826	**VASP**	-3.034
DNAJB6	1.534	**CDCP1**	-4	**PPFIA1**	1.821	**TPM3**	-2.476
		SDCBP	-3.874	**LGALS1**	1.802	**RASA1**	-2.476
		CYP2J2	-3.777	**ETS1**	1.787	**CAT**	-2.164
		ITGB1	-3.766	**MAPK8IP3**	1.765	**EBI3**	-2.121
		E2F5	-3.714	**CDC42**	1.731	**TIMP1**	-2.002
		F2RL1	-3.358	**DYRK1B**	1.716	**ASNS**	-1.844
		DOCK1	-3.257	**LEP**	1.704	**ADAM9**	-1.842
		SSH1	-3.222	**MTA1**	1.691	**FAF1**	-1.798
		NRG1	-3.129	**HS.535028**	1.67	**SMARCB1**	-1.734
		CTNNB1	-3.028	**ILF3**	1.652	**FAM188A**	-1.722
		CD151	-2.924	**DIDO1**	1.63	**WDR44**	-1.679
		CCL20	-2.862	**VEGFB**	1.627	**GNAI1**	-1.618
		SERPINE1	-2.835	**CD24**	1.6	**PARK2**	-1.575
		BMPR2	-2.784	**CDC25B**	1.597	**IGFBP4**	-1.559
		AFAP1	-2.7	**SHC1**	1.58	**ARHGAP2**	-1.519
		SMAD3	-2.627	**HES6**	1.52	**PALLD**	-1.512
		EIF2AK2	-2.549			**KRT19**	-1.511
		ACTA2	-2.542				
		CAV1	-2.515				
		CD81	-2.482				
		JAK1	-2.43				
		TNF	-2.402				
		CD44	-2.382				
		HAS3	-2.356				
		HS.128753	-2.337				
		CTTN	-2.309				
		MAP3K7	-2.285				
		CX3CL1	-2.264				
		NOTCH2	-2.247				
		MAP2K1	-2.213				

		ITGA6	-2.21				
		FOSL1	-2.21				
		LIN28B	-2.204				
		MERTK	-2.188				
		SLC12A2	-2.114				
		CUL4A	-2.051				
		RAB21	-2.018				
		PTHLH	-2.018				
		VGF	-2.002				
		S1PR3	-1.994				
		GAB2	-1.989				
		HNRPK	-1.981				
		ADAM17	-1.975				
		ARFGEF1	-1.959				
		CYR61	-1.957				
		TNFAIP8	-1.942				
		RPS6KA3	-1.939				
		ITGAV	-1.924				
		DAB2	-1.919				
		SFN	-1.916				
		PRMT6	-1.908				
		KPNA2	-1.905				
		PRKCA	-1.889				
		FOXQ1	-1.88				
		MAPK1	-1.879				
		ITGA2	-1.873				
		JUN	-1.871				
		MAP3K8	-1.871				
		KRAS	-1.824				
		SMAD4	-1.8				
		VEGFC	-1.796				
		MIR21	-1.795				
		ADAM10	-1.774				
		ALCAM	-1.768				
		ANKRD28	-1.757				
		FNBP1L	-1.753				
		APP	-1.751				
		IQGAP1	-1.751				
		WWTR1	-1.746				
		PLD1	-1.734				
		VAMP7	-1.733				
		MAP4K4	-1.729				
		FER	-1.721				
		GOLPH3	-1.717				
		CD40	-1.717				
		PTGER4	-1.712				
		TRIO	-1.712				
		USP9X	-1.709				
		F2R	-1.706				
		GNAI3	-1.699				
		HS.4988	-1.698				
		AURKA	-1.697				

		ERBB2	-1.683				
		CDKN1A	-1.678				
		ZEB1	-1.67				
		HAS2	-1.67				
		EGFR	-1.661				
		ITPR1	-1.653				
		RHOB	-1.651				
		WASL	-1.648				
		PAK2	-1.645				
		RANBP9	-1.641				
		MTDH	-1.623				
		ANXA2	-1.619				
		LMO7	-1.61				
		FYN	-1.601				
		ETV4	-1.6				
		ECT2	-1.593				
		CAPN2	-1.585				
		LTBP2	-1.58				
		SLC2A1	-1.571				
		JAK2	-1.568				
		CSE1L	-1.566				
		MUC1	-1.556				
		AKAP11	-1.551				
		HGF	-1.545				
		CTNNAL1	-1.526				
		TCF12	-1.522				
		ARRB2	-1.519				
		FRAP1	-1.518				
		CXCR4	-1.515				
		ACSL4	-1.515				
		CTBP2	-1.508				
		WASF1	-1.507				
		DNM2	-1.506				
		EZH2	-1.503				

Supplemental Table B3. Sp4-regulated associated with growth inhibition, cell death and inhibition of migration/invasion after Sp4 knockdown: expected and inversely regulated genes.

A.

Expected				**Inversely related**			
decreased cell proliferation				**increased cell proliferation**			
up-genes (68)	fold change	**down genes (447)**	fold change	**up-genes (131)**	fold change	**down genes (221)**	fold change
TXNIP	4.646	**EGR1**	-13.207	**LMNA**	10.206	**DCBLD2**	-6.867
CDCA4	3.479	**RRM2**	-10.183	**NCOR2**	3.634	**STAT1**	-5.293
RNH1	3.269	**FOS**	-5.995	**HMGB1**	3.379	**PLOD2**	-4.87
HMOX1	3.189	**PDE5A**	-5.765	**C1QBP**	3.093	**LIPA**	-4.506
NUMA1	2.972	**CTGF**	-5.348	**HMGB2**	3.081	**ARRDC3**	-3.84
EI24	2.961	**BIRC3**	-4.833	**RCE1**	3.003	**EIF4G2**	-3.785
TCF3	2.595	**NCSTN**	-4.492	**H2AFX**	2.91	**ITGA6**	-3.665
MSX1	2.469	**DKK1**	-4.291	**CBX7**	2.91	**TGFBR3**	-3.607

MXD4	2.454	**DUSP6**	-4.027	**SERPINH1**	2.644	**PNPT1**	-3.293
UCP2	2.453	**LIN28B**	-3.856	**PTPMT1**	2.629	**TRIB1**	-3.286
EIF1	2.369	**GOLM1**	-3.835	**SUMO2**	2.39	**AKAP12**	-3.279
TIMP2	2.349	**RPS6KA3**	-3.8	**LRPAP1**	2.336	**LIFR**	-3.193
RALBP1	2.288	**BIRC2**	-3.744	**NOLC1**	2.264	**TFPI**	-3.143
GPR56	2.251	**IL6ST**	-3.727	**CDCA5**	2.226	**SPRY1**	-3.016
GEMIN2	2.233	**AFAP1**	-3.709	**NOP58**	2.209	**EGR2**	-3.01
SH2B3	2.221	**DDX58**	-3.697	**ARF1**	2.207	**CHEK2**	-2.983
DNAJA3	2.179	**CCNB1**	-3.663	**ACIN1**	2.203	**B3GNT5**	-2.962
ACTN4	2.107	**CXCL8**	-3.59	**EMP1**	2.173	**ABCC5**	-2.869
SREBF1	2.099	**TFRC**	-3.539	**CDC25B**	2.173	**ASPH**	-2.85
MED25	2.021	**B4GALT6**	-3.49	**CSF2RA**	2.166	**UBE2D3**	-2.821
NFKBIB	2.012	**PPP3CB**	-3.478	**ATP5G1**	2.148	**STX2**	-2.795
NUPR1	1.962	**ADAR**	-3.472	**DEPTOR**	2.125	**ATG5**	-2.746
GADD45A	1.958	**FNDC3B**	-3.381	**ANTXR1**	2.124	**PROS1**	-2.711
SIGIRR	1.929	**IER3**	-3.343	**MCM8**	2.087	**CASP7**	-2.665
NUP62	1.926	**AKIRIN1**	-3.304	**ETS1**	2.086	**CRLF3**	-2.598
HSPA1A/HSPA1	1.919	**AGO2**	-3.198	**EWSR1**	2.075	**SESN1**	-2.596
PCBP4	1.892	**CYR61**	-3.158	**MED1**	2.069	**FRMD6**	-2.595
DAXX	1.89	**PLAU**	-3.072	**TBP**	2.041	**CTNNA1**	-2.582
MUL1	1.885	**MCL1**	-3.012	**SET**	2.028	**TGFBR2**	-2.574
CHD5	1.876	**DICER1**	-2.978	**RPS15A**	2.014	**RRAS**	-2.572
ATF5	1.867	**SERPINE1**	-2.973	**RARA**	2.005	**KRIT1**	-2.557
DNAJB6	1.859	**LBR**	-2.959	**YWHAG**	1.998	**NDFIP1**	-2.509
BCCIP	1.849	**F2R**	-2.95	**H3F3A/H3F3B**	1.971	**ULK2**	-2.479
TSPYL2	1.838	**CTNNB1**	-2.941	**TBC1D8**	1.968	**SPRY2**	-2.451
DIABLO	1.82	**VCL**	-2.927	**NACA**	1.966	**SPRY4**	-2.435
DACT3	1.801	**ERCC1**	-2.888	**MKL1**	1.964	**WIPF1**	-2.423
BMF	1.763	**F2RL1**	-2.884	**ISG15**	1.962	**BMPR2**	-2.396
UXT	1.762	**E2F5**	-2.873	**CREB1**	1.947	**WTAP**	-2.382
COMMD5	1.759	**CD44**	-2.866	**TPD52**	1.936	**RHOB**	-2.37
MTA1	1.752	**DDAH1**	-2.769	**CDK6**	1.935	**ANXA7**	-2.367
EEF1B2	1.731	**ZNF451**	-2.743	**ULK1**	1.915	**PPP2R1B**	-2.359
SSTR2	1.72	**PRKCA**	-2.726	**S100A4**	1.906	**YWHAZ**	-2.351
GPS2	1.703	**GCNT2**	-2.721	**CBS/LOC10272456**	1.897	**PCYOX1**	-2.344
FBLN1	1.69	**ERO1L**	-2.695	**PIP4K2B**	1.893	**H2AFY**	-2.337
STRA13	1.684	**PTHLH**	-2.695	**CORO1B**	1.875	**RBL2**	-2.325
ERF	1.673	**GFM1**	-2.678	**MPRIP**	1.865	**KIFAP3**	-2.307
FBXO2	1.671	**DOCK1**	-2.674	**E2F4**	1.863	**CTH**	-2.295
BNIPL	1.653	**GCH1**	-2.667	**TFF3**	1.851	**SEC23A**	-2.293
HRASLS	1.646	**NBN**	-2.657	**UBE2C**	1.839	**TAP1**	-2.289
FKTN	1.635	**ERO1LB**	-2.639	**HNRNPA2B1**	1.838	**RASA1**	-2.286
ABCC3	1.625	**P4HA1**	-2.623	**CRCP**	1.833	**CAT**	-2.286
DNAJB2	1.616	**STT3B**	-2.621	**LEP**	1.833	**TBK1**	-2.28
GABPB2	1.591	**CITED2**	-2.617	**HOXC6**	1.821	**NFE2L2**	-2.278
EMP3	1.587	**C1GALT1**	-2.611	**RANBP1**	1.82	**PHLDA1**	-2.25
ARID1A	1.575	**ARFGAP3**	-2.61	**FBRS**	1.816	**FAM188A**	-2.249
PDCD4	1.573	**HSPA4**	-2.6	**ID3**	1.814	**ERRFI1**	-2.248
TSPO	1.561	**TXNDC5**	-2.591	**TXLNA**	1.81	**ROCK1**	-2.233
TOP3A	1.56	**RAN**	-2.569	**POLR2L**	1.804	**P4HA2**	-2.217
RPL26	1.555	**PLK2**	-2.565	**PFDN5**	1.804	**CAPRIN1**	-2.198
CCDC85B	1.553	**SGK1**	-2.554	**DOHH**	1.788	**RB1**	-2.193
LILRB1	1.548	**LAMC1**	-2.526	**DCLRE1A**	1.777	**RND3**	-2.173

SRM	1.548	**JAK1**	-2.514	**NDEL1**	1.764	**PLXNA1**	-2.152
ATPIF1	1.54	**NUP98**	-2.507	**NUDT1**	1.759	**RNASEL**	-2.147
NRG2	1.538	**PIGN**	-2.499	**IL18**	1.758	**CAST**	-2.143
RBM10	1.531	**DDX5**	-2.487	**RPS9**	1.749	**FBXO11**	-2.14
TNFRSF25	1.529	**ITGB1**	-2.481	**PPL**	1.749	**IFNL2**	-2.139
MXI1	1.525	**SPTBN1**	-2.478	**STX3**	1.739	**G3BP1**	-2.137
DBI	1.501	**MUC1**	-2.466	**CEP131**	1.737	**TRIM24**	-2.135
		ITGAV	-2.466	**NSMF**	1.72	**JUNB**	-2.12
		STAT5B	-2.464	**MCM10**	1.718	**RBBP4**	-2.111
		TNS3	-2.454	**AHNAK**	1.712	**GEM**	-2.102
		TDG	-2.451	**NAA38**	1.7	**AKTIP**	-2.1
		ICAM1	-2.449	**SRC**	1.692	**ROCK2**	-2.096
		NFKB1	-2.435	**SPTAN1**	1.69	**NFIB**	-2.09
		PIK3CA	-2.434	**BASP1**	1.688	**SERINC3**	-2.087
		CDC16	-2.431	**MCM4**	1.683	**B3GNT2**	-2.08
		FZD7	-2.431	**DUSP12**	1.67	**BTG2**	-2.047
		RYK	-2.422	**ARHGEF1**	1.658	**MXD1**	-2.047
		MFN1	-2.409	**DTL**	1.647	**BHLHE40**	-2.044
		MAP3K7	-2.4	**LGALS1**	1.644	**PARG**	-2.039
		RECK	-2.393	**CD320**	1.643	**TFPI2**	-2.025
		HIF1A	-2.374	**FEN1**	1.635	**DDX3X**	-2.022
		TP53BP1	-2.37	**GJC1**	1.634	**ASNS**	-2.018
		DLGAP5	-2.37	**DAPP1**	1.633	**RAD17**	-2.016
		NFYA	-2.367	**PPP1R15A**	1.632	**CUL2**	-2.005
		EOMES	-2.36	**POLR2F**	1.628	**SMYD2**	-1.999
		NASP	-2.349	**IGBP1**	1.626	**CASP3**	-1.996
		HMGCR	-2.348	**PTP4A2**	1.624	**RNF14**	-1.995
		CYP2J2	-2.339	**THRAP3**	1.622	**RCHY1**	-1.971
		PHIP	-2.336	**MDK**	1.619	**ZMAT3**	-1.967
		CD2AP	-2.323	**SP1**	1.601	**ENPP1**	-1.966
		ARFGEF1	-2.293	**EEF1D**	1.591	**PKP2**	-1.924
		EXTL3	-2.291	**CDC20**	1.59	**PHF14**	-1.919
		KRAS	-2.288	**ARHGEF2**	1.588	**ADAMTS**	-1.918
		CCPG1	-2.286	**CDT1**	1.582	**TRIB3**	-1.914
		EPCAM	-2.282	**FANCD2**	1.58	**TOM1L1**	-1.906
		ACER3	-2.262	**PSMA4**	1.579	**SMAD4**	-1.903
		DNM1L	-2.261	**SHC1**	1.578	**HIAT1**	-1.899
		CSNK1G3	-2.261	**MXD3**	1.577	**PPP2CA**	-1.896
		PRKAA1	-2.258	**DIXDC1**	1.577	**PEX2**	-1.883
		NRG1	-2.255	**PTMA**	1.576	**SMARCA**	-1.882
		LGR4	-2.254	**HSF1**	1.576	**TJP1**	-1.882
		USO1	-2.249	**PTGDS**	1.568	**SYNJ2BP**	-1.879
		PDE4D	-2.248	**CDK4**	1.568	**CCNG2**	-1.87
		ARL5A	-2.235	**S100A6**	1.563	**MAP3K2**	-1.87
		SLC20A1	-2.213	**PPIB**	1.563	**DYRK1A**	-1.869
		BTRC	-2.198	**RALGDS**	1.553	**CSE1L**	-1.869
		HIF1AN	-2.189	**TNFSF14**	1.553	**NACC2**	-1.866
		MAP4K4	-2.187	**PCK2**	1.55	**DNER**	-1.863
		TOP2A	-2.183	**HGS**	1.548	**DLG1**	-1.861
		EPAS1	-2.164	**PIDD1**	1.546	**TP53INP1**	-1.847
		DSG2	-2.162	**SNX12**	1.546	**MERTK**	-1.842
		USP47	-2.16	**CYBA**	1.544	**KIF23**	-1.839

		EIF5A2	-2.158	**TBX1**	1.542	**MORC3**	-1.834
		NAMPT	-2.151	**mir-130**	1.539	**DYNC1H1**	-1.833
		USP18	-2.15	**AKIRIN2**	1.539	**CAPRIN2**	-1.826
		TPR	-2.15	**CNTFR**	1.537	**KLF10**	-1.825
		MAPK13	-2.149	**CISD1**	1.537	**VLDLR**	-1.817
		YES1	-2.148	**SLC7A5**	1.533	**EIF2AK2**	-1.814
		PIK3CB	-2.146	**SLC44A4**	1.53	**STARD13**	-1.81
		DDX17	-2.142	**CKS1B**	1.526	**PTPN14**	-1.791
		FAT4	-2.128	**ACACA**	1.525	**ARID4A**	-1.791
		SLC39A6	-2.126	**PIN1**	1.519	**IFNL3**	-1.79
		ILK	-2.118	**TIMELESS**	1.513	**TES**	-1.785
		PIK3C2A	-2.107	**MFGE8**	1.512	**PTPN12**	-1.782
		CD46	-2.106	**NSA2**	1.512	**GPNMB**	-1.779
		XRCC5	-2.091	**COPS2**	1.511	**ARID4B**	-1.773
		HSPD1	-2.089	**ORAI1**	1.508	**CSNK1A1**	-1.772
		KIF11	-2.088	**SRF**	1.503	**USP16**	-1.77
		TNFRSF10	-2.08	**SH2B1**	1.502	**HOXA5**	-1.757
		PRPS2	-2.077	**SHFM1**	1.501	**CTSV**	-1.756
		TNFAIP8	-2.074			**CPEB1**	-1.755
		ZMYM2	-2.063			**CYLD**	-1.742
		RGPD4	-2.061			**NAB1**	-1.733
		RAB28	-2.06			**DUSP5**	-1.729
		ANGPTL4	-2.057			**RHOH**	-1.729
		MYOF	-2.056			**PDS5B**	-1.72
		RPA1	-2.034			**APBB2**	-1.714
		ITGA2	-2.025			**CDK8**	-1.71
		CXCL1	-2.023			**MCRS1**	-1.705
		BMPR1A	-2.011			**LIMK1**	-1.704
		CDC14B	-2.011			**CAV1**	-1.702
		RALA	-2.005			**TOP1**	-1.702
		DIDO1	-2.004			**SGPL1**	-1.701
		SUV420H1	-2.003			**BACH2**	-1.697
		AMD1	-2			**AJUBA**	-1.693
		GAD1	-1.997			**DVL1**	-1.693
		TADA3	-1.997			**CD83**	-1.691
		REL	-1.995			**SPRED1**	-1.69
		ASCC3	-1.993			**ITPR1**	-1.689
		PRKAR1A	-1.993			**ATG7**	-1.687
		CUL4A	-1.987			**RNF111**	-1.684
		ROR1	-1.987			**ABLIM1**	-1.683
		PLS3	-1.983			**VMP1**	-1.683
		HNRNPK	-1.978			**PAQR3**	-1.679
		SSR1	-1.975			**PHC3**	-1.672
		TSNAX	-1.971			**PPM1A**	-1.669
		STAT4	-1.968			**RGN**	-1.664
		MINA	-1.96			**ST7L**	-1.662
		NFKBIA	-1.955			**FOXN3**	-1.658
		TGIF1	-1.951			**CASK**	-1.656
		ACVR1	-1.951			**ABCC1**	-1.655
		ATP7A	-1.948			**NOTCH2**	-1.649
		ADAM17	-1.945			**TP53BP2**	-1.644
		SDCBP	-1.94			**APPL1**	-1.637

		KDM5B	-1.939			**IFIT3**	-1.613
		ARID3A	-1.939			**BAX**	-1.608
		OPTN	-1.936			**ATP6V0A**	-1.606
		KIF2A	-1.931			**ADIPOR1**	-1.601
		SEMA4D	-1.931			**TSG101**	-1.595
		VEGFC	-1.925			**IFNAR1**	-1.595
		OPA1	-1.923			**FBXL2**	-1.594
		WWTR1	-1.923			**BCAN**	-1.591
		ILF3	-1.919			**ZFP36**	-1.591
		CENPF	-1.915			**KDM3B**	-1.591
		ADAM9	-1.909			**KLK3**	-1.59
		C1GALT1	-1.908			**RANBP9**	-1.589
		DVL3	-1.905			**RAP1GA**	-1.588
		NFAT5	-1.904			**STEAP3**	-1.586
		MAPK1	-1.901			**LATS1**	-1.58
		NAE1	-1.898			**WARS**	-1.579
		MEMO1	-1.896			**HAS3**	-1.579
		IL13RA1	-1.895			**MEN1**	-1.579
		ELMO2	-1.885			**PMEPA1**	-1.574
		NEDD4L	-1.883			**CREG1**	-1.572
		NFIA	-1.882			**TANK**	-1.571
		EXOC5	-1.881			**CD40**	-1.568
		TEAD2	-1.88			**CCNL2**	-1.566
		BPNT1	-1.878			**STK3**	-1.563
		UBE2A	-1.878			**PLSCR1**	-1.562
		CSRP1	-1.874			**LAT2**	-1.561
		MSH2	-1.867			**RPS6KA2**	-1.56
		PAK2	-1.864			**FGFRL1**	-1.556
		PTPN3	-1.864			**PLCL2**	-1.556
		RCAN1	-1.858			**TCF12**	-1.551
		KPNA2	-1.855			**USP10**	-1.55
		ABI2	-1.855			**HERC2**	-1.55
		MALT1	-1.853			**TEX11**	-1.549
		PTPN1	-1.85			**IQGAP1**	-1.548
		BMI1	-1.85			**DAB2**	-1.548
		APP	-1.846			**RASL10A**	-1.543
		GLDC	-1.843			**CDK11B**	-1.539
		MET	-1.834			**IFT88**	-1.535
		WAPAL	-1.832			**PARK2**	-1.534
		LCN2	-1.83			**SIX5**	-1.533
		CCDC88A	-1.827			**RBBP7**	-1.531
		API5	-1.825			**OAS3**	-1.528
		LDHA	-1.823			**CXADR**	-1.527
		FBXW11	-1.823			**KCND2**	-1.527
		AHR	-1.82			**STRN**	-1.527
		RASGRP3	-1.818			**PHLDA2**	-1.525
		IMPACT	-1.816			**KLF13**	-1.52
		PTPN13	-1.816			**CTBP2**	-1.512
		NR2C2	-1.813			**FBXW7**	-1.511
		CTSB	-1.812			**NPDC1**	-1.511
		RUVBL1	-1.811			**EEF1A1**	-1.506
		STAT3	-1.806			**MIB1**	-1.506

		KMT2C	-1.792			**WDR48**	-1.506
		ANXA2	-1.791			**IFNGR1**	-1.504
		NPC1	-1.791			**IFT74**	-1.502
		C8orf44-	-1.79			**TLR6**	-1.501
		BCAT1	-1.789				
		UFL1	-1.789				
		STEAP2	-1.784				
		FUT4	-1.782				
		FOXQ1	-1.778				
		ALCAM	-1.776				
		SPAST	-1.775				
		NOC3L	-1.773				
		PDPK1	-1.769				
		PRMT6	-1.769				
		KIAA1429	-1.766				
		STK38L	-1.765				
		PFKM	-1.761				
		IDE	-1.76				
		WLS	-1.756				
		AURKA	-1.755				
		ARNTL	-1.749				
		TTC5	-1.748				
		ERBB2	-1.74				
		LEPR	-1.739				
		SLC12A2	-1.739				
		PPIA	-1.736				
		ALDOA	-1.736				
		STAM2	-1.734				
		mir-21	-1.733				
		ADM	-1.732				
		IGF1R	-1.729				
		MSI2	-1.729				
		MAP2K5	-1.728				
		POT1	-1.727				
		UBR5	-1.723				
		GNAQ	-1.722				
		FAIM	-1.72				
		EPS8	-1.716				
		FGFR1OP	-1.716				
		GLB1	-1.715				
		STK39	-1.713				
		NRAS	-1.713				
		CCT2	-1.711				
		CHUK	-1.708				
		CTTN	-1.707				
		AMACR	-1.699				
		RBBP9	-1.699				
		AKR1C3	-1.698				
		YAP1	-1.696				
		CDK1	-1.693				
		HOXB3	-1.691				
		LTB	-1.691				

		PTPN11	-1.688				
		RECQL	-1.687				
		FDFT1	-1.687				
		MSH3	-1.686				
		HGF	-1.685				
		NFKB2	-1.685				
		HSP90B1	-1.683				
		BIRC6	-1.682				
		CDK13	-1.679				
		ACTB	-1.678				
		PALLD	-1.676				
		FKBP1A	-1.676				
		SMC3	-1.675				
		PLD1	-1.675				
		BUB3	-1.674				
		TGIF2	-1.674				
		CD164	-1.673				
		CUL1	-1.673				
		MDM2	-1.672				
		ADNP2	-1.671				
		CCNA2	-1.67				
		NCOA2	-1.666				
		KIF3A	-1.666				
		ABCB7	-1.665				
		TASP1	-1.664				
		TNF	-1.663				
		TIRAP	-1.662				
		FUT8	-1.661				
		NUMB	-1.66				
		HDAC8	-1.659				
		RIPK1	-1.655				
		CASP8	-1.652				
		RAB1A	-1.648				
		EXOC4	-1.647				
		HLA-DMB	-1.646				
		PRKX	-1.642				
		COPS4	-1.639				
		MAP2K1	-1.639				
		SLC30A6	-1.638				
		OSBPL1A	-1.637				
		HEYL	-1.637				
		CDK2	-1.635				
		DNAJA2	-1.635				
		PSMC2	-1.634				
		CD151	-1.634				
		VGF	-1.633				
		EIF4A1	-1.632				
		PKM	-1.63				
		RAB11A	-1.629				
		EPHB2	-1.628				
		CDK5	-1.627				
		EBI3	-1.623				

		TAF9B	-1.622				
		MAPK6	-1.62				
		CCNE1	-1.619				
		PIK3R1	-1.619				
		RAD50	-1.617				
		PGK1	-1.616				
		RHOQ	-1.616				
		RAB5A	-1.615				
		FGF2	-1.613				
		IL11RA	-1.611				
		CCND1	-1.609				
		USP1	-1.608				
		TTLL7	-1.608				
		DMTF1	-1.607				
		ARFGEF2	-1.607				
		AGO4	-1.606				
		KIF20B	-1.605				
		MAP3K4	-1.604				
		SYNM	-1.604				
		ALG14	-1.603				
		MYD88	-1.602				
		MAPK9	-1.601				
		SLC52A1	-1.601				
		BTN3A1	-1.601				
		KIF3C	-1.6				
		EEA1	-1.599				
		GLCE	-1.597				
		EXT1	-1.596				
		ECT2	-1.596				
		DDR1	-1.595				
		HUWE1	-1.594				
		TACC2	-1.593				
		SRSF5	-1.589				
		TRAF5	-1.589				
		RAP1B	-1.588				
		CDH1	-1.587				
		ARHGAP2	-1.587				
		SS18	-1.585				
		PRDX3	-1.584				
		PTPRA	-1.583				
		SPP1	-1.581				
		ATF6	-1.579				
		LATS2	-1.579				
		CALCRL	-1.578				
		TCP1	-1.577				
		MBP	-1.575				
		AGGF1	-1.574				
		CNOT6	-1.572				
		FZD4	-1.571				
		NEK2	-1.57				
		FER	-1.569				
		ASPM	-1.567				
		METAP2	-1.567				

		BMP6	-1.566				
		STAM	-1.566				
		E2F6	-1.565				
		KCNMA1	-1.565				
		PLAG1	-1.565				
		HK2	-1.563				
		TRIB2	-1.563				
		MORF4L1	-1.561				
		TEAD4	-1.56				
		CLCN3	-1.558				
		STK17A	-1.558				
		CSNK2A1	-1.557				
		CHKA	-1.557				
		MAP1B	-1.557				
		RALB	-1.556				
		ACSL4	-1.555				
		SHMT1	-1.554				
		SMO	-1.553				
		FASN	-1.553				
		TNFRSF12	-1.55				
		LIG4	-1.55				
		E2F3	-1.549				
		TRPC1	-1.549				
		CAPN2	-1.548				
		ITGB4	-1.547				
		PDGFC	-1.546				
		ASAH1	-1.545				
		CASP6	-1.545				
		AGO3	-1.545				
		RLIM	-1.544				
		MCFD2	-1.544				
		ID4	-1.543				
		PLCE1	-1.543				
		PRKD3	-1.54				
		SLC30A1	-1.539				
		HOXB4	-1.538				
		PTGER4	-1.538				
		ACER2	-1.536				
		EGFR	-1.535				
		CCNE2	-1.535				
		CUL4B	-1.533				
		PREP	-1.532				
		DUSP10	-1.531				
		JAK2	-1.53				
		CCNDBP1	-1.53				
		GOLPH3	-1.529				
		UGCG	-1.527				
		ARRB1	-1.526				
		UBE2N	-1.525				
		RBM3	-1.524				
		RAC1	-1.523				
		SLC12A4	-1.522				

		KIF18A	-1.522				
		COPS6	-1.521				
		MMP25	-1.521				
		LAMB1	-1.521				
		VAPA	-1.519				
		PRKCE	-1.518				
		LCK	-1.516				
		PRKD1	-1.515				
		TIMP1	-1.514				
		KDM2B	-1.513				
		MSRA	-1.512				
		CUX1	-1.51				
		PDF	-1.51				
		STK24	-1.509				
		ARHGAP5	-1.508				
		TPP2	-1.505				
		AKAP13	-1.503				
		EIF4E	-1.502				

B.

Expected				**Inversely related**			
increased cell death				**decreased cell death**			
up-genes (91)	fold change	**down genes (401)**	fold change	**up-genes (101)**	fold change	**down genes (278)**	fold change
TXNIP	4.646	**RRM2**	-10.183	**ARID3B**	3.667	**EGR1**	-13.207
NCOR2	3.634	**SLC4A7**	-5.397	**HMOX1**	3.189	**CD69**	-10.009
SMARCC	3.407	**CTGF**	-5.348	**EI24**	2.961	**FOS**	-5.995
HMGB1	3.379	**BIRC3**	-4.833	**RGS10**	2.924	**PDE5A**	-5.765
HMGB2	3.081	**NCSTN**	-4.492	**STX8**	2.918	**STAT1**	-5.293
NUMA1	2.972	**SCARB2**	-4.157	**MEF2D**	2.859	**LIPA**	-4.506
H2AFX	2.91	**DCAF7**	-4.08	**SERPINH1**	2.644	**DKK1**	-4.291
HS1BP3	2.75	**RRM2B**	-3.882	**PTPMT1**	2.629	**DUSP6**	-4.027
TCF3	2.595	**RPS6KA3**	-3.8	**MSX1**	2.469	**ANTXR2**	-3.813
ITGB3BP	2.534	**EIF4G2**	-3.785	**LMO4**	2.36	**AFAP1**	-3.709
BOK	2.277	**NEK6**	-3.76	**TIMP2**	2.349	**DDX58**	-3.697
IL17RD	2.252	**BIRC2**	-3.744	**NUAK1**	2.346	**ITGA6**	-3.665
MYH9	2.208	**IL6ST**	-3.727	**LRPAP1**	2.336	**CCNB1**	-3.663
ACIN1	2.203	**CXCL8**	-3.59	**NOLC1**	2.264	**TGFBR3**	-3.607
DNAJA3	2.179	**NEK7**	-3.481	**SIP1**	2.233	**TFRC**	-3.539
EMP1	2.173	**PPP3CB**	-3.478	**CREBBP**	2.207	**IER3**	-3.343
CSF2RA	2.166	**ADAR**	-3.472	**DMC1**	2.184	**IFIH1**	-3.322
DPM3	2.15	**TRIB1**	-3.286	**DAG1**	2.155	**PNPT1**	-3.293
ANTXR1	2.124	**AKAP12**	-3.279	**DEPDC6**	2.125	**OLR1**	-3.236
SREBF1	2.099	**AGO2**	-3.198	**ACO2**	2.101	**TFPI**	-3.143
ETS1	2.086	**NSF**	-3.186	**EWSR1**	2.075	**GULP1**	-3.08
PPARBP	2.069	**CYR61**	-3.158	**RPS24**	2.067	**CHEK2**	-2.983
SET	2.028	**CCT6A**	-3.12	**TBP**	2.041	**F2R**	-2.95
BRMS1	2.019	**PLAU**	-3.072	**KRT8**	2.034	**SLC25A24**	-2.885
NFKBIB	2.012	**MCL1**	-3.012	**NME2**	2.011	**F2RL1**	-2.884

PTRH2	2.006	**EGR2**	-3.01	**RARA**	2.005	**GFPT1**	-2.835
IL10	1.979	**LDLR**	-2.978	**BCL2L12**	2.002	**SCP2**	-2.828
ISG15	1.962	**DICER1**	-2.978	**YWHAG**	1.998	**PPP2R5A**	-2.777
NUPR1	1.962	**SERPINE1**	-2.973	**PLEC1**	1.99	**ATG5**	-2.746
GADD45A	1.958	**WEE1**	-2.951	**TAOK3**	1.972	**ZAK**	-2.735
CCDC86	1.944	**TBL1XR1**	-2.941	**INTS1**	1.966	**ATG4A**	-2.686
CDK6	1.935	**CTNNB1**	-2.941	**MKL1**	1.964	**DOCK1**	-2.674
DAXX	1.89	**VCL**	-2.927	**CREB1**	1.947	**GCH1**	-2.667
MPRIP	1.865	**ERCC1**	-2.888	**TPD52**	1.936	**CASP7**	-2.665
PRELID1	1.864	**ABCC5**	-2.869	**SIGIRR**	1.929	**ZFYVE16**	-2.644
DIABLO	1.82	**CD44**	-2.866	**NUP62**	1.926	**ATP2A2**	-2.641
ID3	1.814	**MTM1**	-2.838	**HSPA1B**	1.919	**ZMYND11**	-2.627
E2F2	1.782	**CDCP1**	-2.796	**S100A4**	1.906	**FRMD6**	-2.595
TGFBI	1.774	**APH1B**	-2.791	**CAMK2G**	1.902	**CTNNA1**	-2.582
BMF	1.763	**PRKCA**	-2.726	**CBS**	1.897	**CTSL1**	-2.581
LAMP1	1.76	**PROS1**	-2.711	**ATF5**	1.867	**DRAM1**	-2.533
MBD4	1.758	**PTHLH**	-2.695	**E2F4**	1.863	**JAK1**	-2.514
IL18	1.758	**NBN**	-2.657	**DNAJB6**	1.859	**CCNC**	-2.508
RASSF6	1.755	**BBS2**	-2.643	**TRIAP1**	1.858	**DDX5**	-2.487
FAU	1.747	**CITED2**	-2.617	**TFF3**	1.851	**ITGB1**	-2.481
ADRM1	1.734	**HSPA4**	-2.6	**EBAG9**	1.847	**ULK2**	-2.479
MRPL41	1.733	**TXNDC5**	-2.591	**UBE2C**	1.839	**SPTBN1**	-2.478
UBE2L3	1.725	**TGFBR2**	-2.574	**LEP**	1.833	**ICAM1**	-2.449
SSTR2	1.72	**RRAS**	-2.572	**HOXC6**	1.821	**PDCD6IP**	-2.435
GPS2	1.703	**PLK2**	-2.565	**NCOA6**	1.814	**WASPIP**	-2.423
TNFAIP8L	1.667	**KIF14**	-2.559	**CDR2L**	1.812	**RECK**	-2.393
NRGN	1.663	**SGK1**	-2.554	**MAD2L2**	1.799	**WTAP**	-2.382
BNIPL	1.653	**FANCL**	-2.533	**DDIT4**	1.791	**RHOB**	-2.37
CAMLG	1.651	**MUC1**	-2.466	**NDEL1**	1.764	**MX1**	-2.358
HRASLS	1.646	**ITGAV**	-2.466	**UXT**	1.762	**LGALS8**	-2.322
LGALS1	1.644	**STAT5B**	-2.464	**NUDT1**	1.759	**PRKDC**	-2.306
AKAP1	1.637	**APIP**	-2.458	**HSPBP1**	1.751	**PDCD2**	-2.3
PPP1R15	1.632	**SPRY2**	-2.451	**TUB**	1.73	**CTH**	-2.295
EGLN2	1.628	**NCEH1**	-2.44	**MCM10**	1.718	**KRAS**	-2.288
CCAR1	1.618	**NFKB1**	-2.435	**CENPB**	1.703	**RASA1**	-2.286
DEDD2	1.611	**PIK3CA**	-2.434	**LSMD1**	1.7	**DNM1L**	-2.261
SIVA1	1.605	**MFN1**	-2.409	**SRC**	1.692	**HS.551128**	-2.255
STX1A	1.6	**MAP3K7**	-2.4	**NUF2**	1.692	**PHLDA1**	-2.25
ATN1	1.594	**LIN7C**	-2.395	**FBLN1**	1.69	**XPR1**	-2.241
CDC20	1.59	**VAMP3**	-2.388	**WDR4**	1.685	**MAP4K4**	-2.187
EMP3	1.587	**HSD17B4**	-2.386	**TNRC6A**	1.684	**TOP2A**	-2.183
CACNB3	1.582	**SORL1**	-2.379	**SCG5**	1.681	**PARP14**	-2.165
CDT1	1.582	**UBA3**	-2.374	**ERF**	1.673	**DSG2**	-2.162
SHC1	1.578	**HIF1A**	-2.374	**FBXO2**	1.671	**MAPK13**	-2.149
PDCD4	1.573	**TOP2B**	-2.371	**ARHGEF1**	1.658	**YES1**	-2.148
HIST1H1C	1.57	**TP53BP1**	-2.37	**FEN1**	1.635	**RNASEL**	-2.147
PTGDS	1.568	**PLS1**	-2.369	**GJC1**	1.634	**GPR37**	-2.137
CDK4	1.568	**ANXA7**	-2.367	**STXBP1**	1.632	**AP1G1**	-2.128
S100A6	1.563	**NFYA**	-2.367	**ABCC3**	1.625	**RFK**	-2.125
PPIB	1.563	**LRP8**	-2.361	**CHMP4B**	1.625	**RBBP4**	-2.111
TNFSF14	1.553	**EOMES**	-2.36	**PTP4A2**	1.624	**QKI**	-2.096
BRCA1	1.549	**PPP2R1B**	-2.359	**MDK**	1.619	**ROCK2**	-2.096
LILRB1	1.548	**YWHAZ**	-2.351	**CCBE1**	1.617	**SERINC3**	-2.087

LRDD	1.546	**HMGCR**	-2.348	**DNAJB2**	1.616	**TNFRSF10B**	-2.08
BNIP3L	1.545	**ABCE1**	-2.342	**TAF10**	1.614	**SEC23B**	-2.071
CYBA	1.544	**CYP2J2**	-2.339	**DAD1**	1.602	**AIFM1**	-2.053
MIR130A	1.539	**PHIP**	-2.336	**SP1**	1.601	**BPTF**	-2.043
DFFA	1.538	**RBL2**	-2.325	**EEF1D**	1.591	**SH3RF1**	-2.042
TNFRSF2	1.529	**CD2AP**	-2.323	**ARHGEF2**	1.588	**PARG**	-2.039
PIN1	1.519	**FBXL5**	-2.308	**APRT**	1.582	**TFPI2**	-2.025
FAM162A	1.519	**KIFAP3**	-2.307	**FANCD2**	1.58	**DDX3X**	-2.022
NME4	1.512	**CAT**	-2.286	**PRDX5**	1.58	**PKN2**	-2.021
ORAI1	1.508	**EPCAM**	-2.282	**MXD3**	1.577	**GNA12**	-2.02
PRPS1	1.507	**MBTPS1**	-2.282	**PTMA**	1.576	**SOAT1**	-2.018
MUTYH	1.506	**TBK1**	-2.28	**UBQLN1**	1.576	**PSEN2**	-2.009
KIAA0831	1.505	**VPS13A**	-2.28	**HSF1**	1.576	**RFWD2**	-2.005
		NFE2L2	-2.278	**ARID1A**	1.575	**CASP3**	-1.996
		CPEB4	-2.26	**PCK2**	1.55	**WDR19**	-1.995
		PRKAA1	-2.258	**NRG2**	1.538	**PRKAR1A**	-1.993
		PEX11B	-2.256	**CNTFR**	1.537	**IL1R1**	-1.993
		TXNRD1	-2.255	**FANCE**	1.536	**ING3**	-1.992
		LGR4	-2.254	**GADD45GIP1**	1.527	**CCDC109A**	-1.978
		PDE4D	-2.248	**TNPO2**	1.526	**STAT4**	-1.968
		STK40	-2.243	**MFGE8**	1.512	**ZMAT3**	-1.967
		BCL2L13	-2.23	**NEDD8**	1.51	**SP110**	-1.952
		SLC20A1	-2.213	**SRF**	1.503	**GSR**	-1.919
		SERP1	-2.21			**ADAMTS1**	-1.918
		BTRC	-2.198			**GMFB**	-1.917
		CAPRIN1	-2.198			**TRIB3**	-1.914
		ATP2B4	-2.195			**SMAD4**	-1.903
		SIAH2	-2.195			**NAE1**	-1.898
		RB1	-2.193			**PPP2CA**	-1.896
		CERS6	-2.187			**ALDH1A3**	-1.89
		IPPK	-2.185			**MCOLN3**	-1.888
		FIGNL1	-2.183			**TLR1**	-1.876
		RND3	-2.173			**CSE1L**	-1.869
		EPAS1	-2.164			**MSH2**	-1.867
		USP47	-2.16			**HS.531457**	-1.866
		NAMPT	-2.151			**PAK2**	-1.864
		USP18	-2.15			**MALT1**	-1.853
		TPM3	-2.15			**PTPN1**	-1.85
		TPR	-2.15			**TP53INP1**	-1.847
		GRB10	-2.147			**ZFAND5**	-1.846
		PIK3CB	-2.146			**APP**	-1.846
		CAST	-2.143			**SNAP25**	-1.843
		TMED10	-2.141			**OAS1**	-1.841
		IFNL2	-2.139			**MLKL**	-1.84
		TRIM24	-2.135			**KIF1B**	-1.837
		SUN1	-2.135			**CREBL2**	-1.837
		FAT4	-2.128			**IRF7**	-1.831
		BRE	-2.127			**LCN2**	-1.83
		MTMR6	-2.126			**CCDC88A**	-1.827
		JUNB	-2.12			**CAPRIN2**	-1.826
		ILK	-2.118			**LDHA**	-1.823
		IREB2	-2.113			**FAF1**	-1.82

		CD46	-2.106			**PTPN13**	-1.816
		GEM	-2.102			**EIF2AK2**	-1.814
		PDE4B	-2.098			**CTSB**	-1.812
		CALM1	-2.093			**TRMT11**	-1.812
		XRCC5	-2.091			**HTRA1**	-1.806
		NFIB	-2.09			**FUT4**	-1.782
		HSPD1	-2.089			**TIA1**	-1.781
		KIF11	-2.088			**UACA**	-1.768
		SLC2A3	-2.08			**IDE**	-1.76
		TNFAIP8	-2.074			**HOXA5**	-1.757
		HLTF	-2.069			**TTC5**	-1.748
		ZMYM2	-2.063			**CYLD**	-1.742
		SPINT1	-2.061			**SGPP1**	-1.742
		RGPD4	-2.061			**TRPS1**	-1.736
		RAB28	-2.06			**STAM2**	-1.734
		MSRB3	-2.059			**TSC22D2**	-1.734
		ANGPTL4	-2.057			**RHOH**	-1.729
		NR2C1	-2.055			**MSI2**	-1.729
		DHX9	-2.054			**MAP2K5**	-1.728
		PIGA	-2.048			**HS.4988**	-1.725
		BTG2	-2.047			**HMOX2**	-1.723
		SORBS2	-2.047			**GNAQ**	-1.722
		MXD1	-2.047			**STAP2**	-1.71
		BHLHE40	-2.044			**TOP1**	-1.702
		RPA1	-2.034			**ZMYM3**	-1.702
		DPYD	-2.033			**SGPL1**	-1.701
		HIPK3	-2.029			**PRKRA**	-1.699
		MTMR2	-2.026			**AKR1C3**	-1.698
		ITGA2	-2.025			**BACH2**	-1.697
		SRI	-2.024			**USP12**	-1.697
		CXCL1	-2.023			**CDC2**	-1.693
		ASNS	-2.018			**LTB**	-1.691
		UBR2	-2.017			**ITPR1**	-1.689
		CUL2	-2.005			**PSMD6**	-1.687
		DIDO1	-2.004			**ATG7**	-1.687
		REL	-1.995			**ERCC3**	-1.682
		SOX21	-1.989			**NSMAF**	-1.68
		GLO1	-1.987			**FKBP1A**	-1.676
		ROR1	-1.987			**KLRF1**	-1.676
		HNRNPK	-1.978			**KIAA1468**	-1.676
		ADH5	-1.967			**PPM1A**	-1.669
		NFKBIA	-1.955			**TNF**	-1.663
		ATP7A	-1.948			**TIRAP**	-1.662
		SERPINI1	-1.945			**NUMB**	-1.66
		ADAM17	-1.945			**RIPK1**	-1.655
		TAX1BP1	-1.94			**ADRB1**	-1.654
		OPTN	-1.936			**CASP8**	-1.652
		ARF4	-1.928			**NOTCH2**	-1.649
		VEGFC	-1.925			**RAB1A**	-1.648
		OPA1	-1.923			**CKAP2**	-1.645
		ILF3	-1.919			**TP53BP2**	-1.644
		CD47	-1.916			**APPL1**	-1.637

		CENPF	-1.915			**CDK2**	-1.635
		SRXN1	-1.911			**VGF**	-1.633
		ATP2B1	-1.909			**SPIN1**	-1.633
		NFAT5	-1.904			**HIP2**	-1.631
		MAPK1	-1.901			**EPHB2**	-1.628
		PAFAH2	-1.901			**CDK5**	-1.627
		CES1	-1.891			**WWC3**	-1.623
		SNX7	-1.883			**CCNE1**	-1.619
		SMARCA1	-1.882			**TMEM158**	-1.616
		TEAD2	-1.88			**IL11RA**	-1.611
		SMARCA5	-1.879			**MAP3K8**	-1.61
		MAP3K2	-1.87			**BAX**	-1.608
		RCAN1	-1.858			**SENP2**	-1.606
		KPNA2	-1.855			**ANKRD1**	-1.602
		BMI1	-1.85			**MYD88**	-1.602
		MERTK	-1.842			**MAPK9**	-1.601
		EXOC2	-1.841			**NIF3L1**	-1.597
		MET	-1.834			**M6PR**	-1.596
		DYNC1H1	-1.833			**ECT2**	-1.596
		WAPAL	-1.832			**IFNAR1**	-1.595
		API5	-1.825			**BID**	-1.594
		AHR	-1.82			**FBXL2**	-1.594
		RANBP2	-1.818			**HUWE1**	-1.594
		EFEMP1	-1.814			**DEDD**	-1.592
		NR2C2	-1.813			**TBCCD1**	-1.592
		HEXB	-1.808			**ZFP36**	-1.591
		STAT3	-1.806			**RANBP9**	-1.589
		ANXA2	-1.791			**SORT1**	-1.588
		NPC1	-1.791			**MAP3K9**	-1.587
		C8orf44-	-1.79			**STEAP3**	-1.586
		GPHN	-1.79			**MOAP1**	-1.586
		SON	-1.786			**PTPRA**	-1.583
		YME1L1	-1.784			**ATP1A1**	-1.583
		HDAC9	-1.783			**LATS1**	-1.58
		DSP	-1.776			**ATF6**	-1.579
		ALCAM	-1.776			**HAS3**	-1.579
		HADHA	-1.772			**LATS2**	-1.579
		SQSTM1	-1.771			**MEN1**	-1.579
		PDPK1	-1.769			**PANX1**	-1.578
		PFKM	-1.761			**MBP**	-1.575
		CTSV	-1.756			**MBD1**	-1.574
		LIMS1	-1.755			**PMEPA1**	-1.574
		AURKA	-1.755			**CCNT1**	-1.573
		ERCC5	-1.755			**MYO7A**	-1.573
		MYO6	-1.751			**TFDP2**	-1.571
		LGMN	-1.748			**CD40**	-1.568
		XPO1	-1.741			**HOXA13**	-1.567
		ERBB2	-1.74			**BMP6**	-1.566
		LEPR	-1.739			**CCNL2**	-1.566
		SLC12A2	-1.739			**SUB1**	-1.566
		PPIA	-1.736			**STK3**	-1.563
		ALDOA	-1.736			**TRIB2**	-1.563

		YWHAB	-1.733			**SCRIB**	-1.562
		mir-21	-1.733			**PLSCR1**	-1.562
		ADM	-1.732			**RPS6KA2**	-1.56
		ANXA5	-1.73			**STK17A**	-1.558
		IGF1R	-1.729			**CSNK2A1**	-1.557
		POT1	-1.727			**MAP1B**	-1.557
		FAIM	-1.72			**RALB**	-1.556
		TCERG1	-1.719			**TCF12**	-1.551
		BAZ1A	-1.716			**TNFRSF12A**	-1.55
		NRAS	-1.713			**RNPS1**	-1.55
		CKAP5	-1.712			**E2F3**	-1.549
		CDK8	-1.71			**CCBL1**	-1.549
		CHUK	-1.708			**CAPN2**	-1.548
		CTTN	-1.707			**DAB2**	-1.548
		FAM134B	-1.706			**TRIM13**	-1.548
		MCRS1	-1.705			**CASP6**	-1.545
		CAV1	-1.702			**RASL10A**	-1.543
		MTMR9	-1.7			**PERP**	-1.542
		AMACR	-1.699			**PTGER4**	-1.538
		YAP1	-1.696			**ACER2**	-1.536
		DVL1	-1.693			**CUL4B**	-1.533
		PTPN11	-1.688			**TAF1B**	-1.533
		RECQL	-1.687			**ARL8B**	-1.532
		FDFT1	-1.687			**OAS3**	-1.528
		MSH3	-1.686			**ARRB1**	-1.526
		HGF	-1.685			**ZNF148**	-1.526
		NFKB2	-1.685			**RAC1**	-1.523
		HSP90B1	-1.683			**KLF13**	-1.52
		BIRC6	-1.682			**APOBEC3B**	-1.52
		GCLM	-1.681			**NDUFAF1**	-1.52
		VCP	-1.681			**ATP6AP2**	-1.516
		SPG7	-1.679			**CUTL1**	-1.51
		PALLD	-1.676			**STK24**	-1.509
		PLD1	-1.675			**OMA1**	-1.508
		RELB	-1.674			**EEF1A1**	-1.506
		CD164	-1.673			**MIB1**	-1.506
		CUL1	-1.673			**WDR48**	-1.506
		PICK1	-1.672			**IFNGR1**	-1.504
		MDM2	-1.672			**MIR302C**	-1.503
		SLC9A4	-1.672			**PRMT2**	-1.502
		ADNP2	-1.671			**TNFRSF10A**	-1.502
		NCOA2	-1.666			**ATP7B**	-1.502
		KIF3A	-1.666			**TJP2**	-1.501
		ABCB7	-1.665				
		RGN	-1.664				
		TASP1	-1.664				
		ABCC1	-1.655				
		CENPE	-1.652				
		VPS41	-1.652				
		RABGGTA	-1.652				
		ANXA4	-1.649				
		AFG3L2	-1.649				

		YWHAE	-1.645				
		SP3	-1.643				
		ALMS1	-1.643				
		MAP2K1	-1.639				
		HEYL	-1.637				
		CYP26B1	-1.636				
		CD151	-1.634				
		PKM	-1.63				
		PARP16	-1.63				
		NCAPG2	-1.628				
		SLCO1B3	-1.622				
		TAF9B	-1.622				
		PIK3R1	-1.619				
		RAD50	-1.617				
		TMX1	-1.617				
		FGF2	-1.613				
		VAMP2	-1.613				
		IFIT3	-1.613				
		GORASP1	-1.611				
		RNF34	-1.609				
		CCND1	-1.609				
		AGO4	-1.606				
		MAP3K4	-1.604				
		CCT7	-1.603				
		CDC14A	-1.602				
		TSG101	-1.595				
		DDR1	-1.595				
		PON2	-1.593				
		RDH10	-1.593				
		PIAS2	-1.59				
		PSMB8	-1.59				
		KLK3	-1.59				
		PPP1R2	-1.589				
		TRAF5	-1.589				
		RAP1GAP	-1.588				
		RAP1B	-1.588				
		IKBKAP	-1.587				
		CDH1	-1.587				
		PRDX3	-1.584				
		NUPL1	-1.584				
		DPP8	-1.583				
		SLX1A/SLX	-1.582				
		SPP1	-1.581				
		OGG1	-1.58				
		CALCRL	-1.578				
		TCP1	-1.577				
		CERKL	-1.575				
		SELE	-1.574				
		AGGF1	-1.574				
		CCT4	-1.573				
		TANK	-1.571				
		NEK2	-1.57				

		FER	-1.569				
		METAP2	-1.567				
		NPHS1	-1.566				
		STAM	-1.566				
		ATP2C1	-1.566				
		E2F6	-1.565				
		KCNMA1	-1.565				
		SETX	-1.565				
		PDE9A	-1.564				
		HK2	-1.563				
		ABCC4	-1.562				
		CLCN3	-1.558				
		CHKA	-1.557				
		FGFRL1	-1.556				
		ACSL4	-1.555				
		HSPA9	-1.555				
		SMO	-1.553				
		FASN	-1.553				
		TMBIM4	-1.551				
		USP10	-1.55				
		LIG4	-1.55				
		TRPC1	-1.549				
		TEX11	-1.549				
		ITGB4	-1.547				
		PDGFC	-1.546				
		ASAH1	-1.545				
		AGO3	-1.545				
		INTS3	-1.541				
		B4GALNT1	-1.54				
		PRKD3	-1.54				
		HOXB4	-1.538				
		ATP11C	-1.538				
		EGFR	-1.535				
		PARD3	-1.535				
		PARK2	-1.534				
		DUSP10	-1.531				
		JAK2	-1.53				
		UGCG	-1.527				
		CXADR	-1.527				
		KCND2	-1.527				
		HSF2	-1.526				
		NEK1	-1.525				
		PPID	-1.524				
		RBM3	-1.524				
		PAF1	-1.523				
		ALDH2	-1.521				
		VAPA	-1.519				
		PRKCE	-1.518				
		ARSB	-1.516				
		GLUD1	-1.515				
		PRKD1	-1.515				
		TIMP1	-1.514				

		KDM2B	-1.513				
		CTBP2	-1.512				
		PSMC1	-1.511				
		FBXW7	-1.511				
		RAD18	-1.51				
		LCMT1	-1.508				
		TPP2	-1.505				
		LYNX1	-1.504				
		KRT18	-1.503				
		EIF4E	-1.502				
		TLR6	-1.501				

C.

Expected				**Inversely related**			
decreased migration				**increased migration**			
up-genes (12)	fold change	**down genes (123)**	fold change	**up-genes (19)**	fold change	**down genes (27)**	fold change
HMOX1	3.189	**EGR1**	-13.207	**C1QBP**	3.093	**STAT1**	-5.293
TIMP2	2.349	**CTGF**	-5.348	**PPFIA1**	2.365	**ARRDC3**	-3.84
MYH9	2.208	**LIN28B**	-3.856	**ARF1**	2.207	**LAMC2**	-3.579
DNAJA3	2.179	**RPS6KA3**	-3.8	**DPAGT1**	2.185	**SPRY2**	-2.451
KRT8	2.034	**AFAP1**	-3.709	**CDC25B**	2.173	**RASA1**	-2.286
BRMS1	2.019	**DDX58**	-3.697	**ACTN4**	2.107	**CAT**	-2.286
DNAJB6	1.859	**ITGA6**	-3.665	**ETS1**	2.086	**FAM188A**	-2.249
CMTM8	1.717	**CXCL8**	-3.59	**S100A4**	1.906	**TPM3**	-2.15
SP1	1.601	**AKAP11**	-3.208	**HNRNPA2B1**	1.838	**ASNS**	-2.018
PDCD4	1.573	**CYR61**	-3.158	**LEP**	1.833	**CD47**	-1.916
BRCA1	1.549	**PLAU**	-3.072	**MTA1**	1.752	**ADAM9**	-1.909
mir-130	1.539	**SERPINE1**	-2.973	**SRC**	1.692	**PTPN1**	-1.85
		F2R	-2.95	**ARHGEF1**	1.658	**TP53INP1**	-1.847
		CTNNB1	-2.941	**LGALS1**	1.644	**FAF1**	-1.82
		F2RL1	-2.884	**PTP4A2**	1.624	**GNAI1**	-1.796
		E2F5	-2.873	**MDK**	1.619	**SACM1L**	-1.725
		CD44	-2.866	**SHC1**	1.578	**PALLD**	-1.676
		CDCP1	-2.796	**MAPK8IP3**	1.575	**EBI3**	-1.623
		PRKCA	-2.726	**ORAI1**	1.508	**FBXL2**	-1.594
		PTHLH	-2.695			**WDR44**	-1.592
		DOCK1	-2.674			**RAP1GAP**	-1.588
		FNBP1L	-2.614			**CDH1**	-1.587
		JAK1	-2.514			**MEN1**	-1.579
		ITGB1	-2.481			**BMP6**	-1.566
		MUC1	-2.466			**PARK2**	-1.534
		ITGAV	-2.466			**PRKD1**	-1.515
		MAP3K7	-2.4			**TIMP1**	-1.514
		BMPR2	-2.396				
		HIF1A	-2.374				
		RHOB	-2.37				
		CYP2J2	-2.339				
		ARFGEF1	-2.293				
		KRAS	-2.288				

		NRG1	-2.255				
		MAP4K4	-2.187				
		ILK	-2.118				
		NES	-2.109				
		TNFAIP8	-2.074				
		ITGA2	-2.025				
		GNA12	-2.02				
		ANKRD28	-2.007				
		DIDO1	-2.004				
		CUL4A	-1.987				
		ROR1	-1.987				
		HNRNPK	-1.978				
		ADAM17	-1.945				
		SDCBP	-1.94				
		VEGFC	-1.925				
		GNAI3	-1.924				
		WWTR1	-1.923				
		ILF3	-1.919				
		USP9X	-1.909				
		SMAD4	-1.903				
		MAPK1	-1.901				
		CSE1L	-1.869				
		PAK2	-1.864				
		KPNA2	-1.855				
		BMI1	-1.85				
		APP	-1.846				
		MERTK	-1.842				
		MET	-1.834				
		CCDC88A	-1.827				
		EIF2AK2	-1.814				
		RUVBL1	-1.811				
		LTBP2	-1.807				
		STAT3	-1.806				
		ANXA2	-1.791				
		FOXQ1	-1.778				
		ALCAM	-1.776				
		PRMT6	-1.769				
		AURKA	-1.755				
		ERBB2	-1.74				
		SLC12A2	-1.739				
		mir-21	-1.733				
		IGF1R	-1.729				
		MGAT5	-1.725				
		NRAS	-1.713				
		CTTN	-1.707				
		LIMK1	-1.704				
		CAV1	-1.702				
		ITPR1	-1.689				
		PTPN11	-1.688				
		HGF	-1.685				
		VCP	-1.681				
		PLD1	-1.675				

		TNF	-1.663				
		CASP8	-1.652				
		NOTCH2	-1.649				
		PODXL	-1.639				
		MAP2K1	-1.639				
		CD151	-1.634				
		VGF	-1.633				
		EPHB2	-1.628				
		CDK5	-1.627				
		FGF2	-1.613				
		MAP3K8	-1.61				
		SYNM	-1.604				
		ECT2	-1.596				
		IFNAR1	-1.595				
		RAB21	-1.592				
		RANBP9	-1.589				
		RAP1B	-1.588				
		SPP1	-1.581				
		HAS3	-1.579				
		FER	-1.569				
		CD40	-1.568				
		RALB	-1.556				
		ACSL4	-1.555				
		TCF12	-1.551				
		TMBIM4	-1.551				
		WASL	-1.55				
		CAPN2	-1.548				
		IQGAP1	-1.548				
		DAB2	-1.548				
		PTGER4	-1.538				
		EGFR	-1.535				
		JAK2	-1.53				
		GOLPH3	-1.529				
		ARRB1	-1.526				
		RAC1	-1.523				
		LCK	-1.516				
		CTBP2	-1.512				
		CTNNAL1	-1.504				

Supplemental Table B4. A list of antibodies, primers and siRNAs that were used and their manufacturers in Chapter VIII.

Antibodies	**Company**
β1-Integrin	Santa Cruz Biotechnology (Santa Cruz, CA)
Sp3	
Sp4	
EGFR	
ZBTB4	
p300	
Secondary mouse IgG-HRP	
normal rabbit IgG	
Secondary goat IgG-HRP	
β4-Integrin	Cell Signaling Technology (Danvers, MA)
α5-Integrin	
α6-Integrin	
FAK	
p-FAK	
c-PARP	
c-Caspase3	
survivin	
c-myc	
secondary rabbit-HRP	
Sp1	Abcam (Cambridge, MA)
Histone H3K27 Ab	
Histone H3K4 Ab	
ZBTB10	Bethyl Laboratories (Montgomery, TX
RNA polymerase II	Active Motif (Carlsbad, CA)
Histone H4K16	
NR4A1	Novus Biologicals (Littleton, CO)

Supplemental Table B4 (Continued).

β-Actin	Sigma-Aldrich (St. Louis, MO)

Primers and siRNA		Company
c-Myc ChIP primer sequences		
c-Myc-ChIP-F	GCC CTT TCC CCA GCC TTA GC	Sigma-Aldrich (St. Louis, MO)
c-Myc-ChIP-R	AAC CGC ATC CTT GTC CTG TGA GTA	
α6-Integrin ChIP primer sequences		
ITGA6-ChIP-F	CTCTGTGCTACTCGGCAAC	Sigma-Aldrich (St. Louis, MO)
ITGA6-ChIP-R	GTT CTCTGGAGACTCGCAGG	
β4-Integrin ChIP primer sequences		
ITGB4-ChIP-F	CCG GCG GCG GCA CCC AGC TCC T	Sigma-Aldrich (St. Louis, MO)
ITGB4-ChIP-R	CCT CTT CCT CCT CGG GGC GGA	
α5-Integrin ChIP primer sequences		

Supplemental Table B4 (Continued).

ITGA5-ChIP-F	ACAGCAAATCATTCCTACTGAGC	Sigma-Aldrich (St. Louis, MO)
ITGA5-ChIP-R	CCCACTGCCCCTCTAAATGATCT	

β1-Integrin ChIP primer sequences

ITGB1-ChIP-F	TCACCACCCTTCGTGACAC	Sigma-Aldrich (St. Louis, MO)
ITGB1-ChIP-R	GAGATCCTGCATCTCGGAAG	

siRNA sequences		**Company**
siSp1	SASI_Hs02_00363664	Sigma-Aldrich (St. Louis, MO)
siSp3	SASI_Hs01_00211941	
siSp4	SASI_Hs01_00114420	
siNR4A1	SASI_Hs02_00333289	
siGL2 (nonspecific control oligomer)	CGU ACG CGG AAU ACU UCG A	